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THE ROLE OF T-LYMPHOCYTES IN THE PATHOGENESIS OF DENGUE HAEMORRHAGIC FEVER

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November 2006

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ABSTRACT

Dengue is one of the most important human diseases transmitted by an arthropod vector and the incidence of dengue virus infection has been increasing steadily throughout the world. Most infections are asymptomatic but a subset of patients experience a potentially fatal shock syndrome characterised by plasma leakage. Generally attributed to the phenomenon of antibody-dependent enhancement, recent observations indicate that T cells may influence the development of this disease and it is this arm of the immune response to dengue this thesis examines.

It starts by describing the production of novel HLA "tetramers" required for the work and then examines the role played by CD8+ cytotoxic T lymphocytes (CTL). This work demonstrates that CTL showing high level cross reactivity between dengue serotypes tend to exhibit high avidity and can be expanded from blood samples taken during the acute phase of secondary dengue infection. These cells produce much higher levels of both type 1 and certain type 2 cytokines than more serotype specific populations. Highly cross-reactive cells cannot be detected in convalescence when populations demonstrating significant serotype specificity dominate.

The next section of the thesis describes the generation and characterisation of dengue specific CD4+ T cell clones, many of which behave in a highly cross-reactive manner producing large amounts of type 1 cytokines and demonstrating perforin-mediated cytolytic activity associated with an increase in the expression of surface CD107. It debates whether a new epitope has been discovered and discusses the nature of CD4

degeneracy, and its contribution to early cross-reactive immune responses which may facilitate priming of other immune system components.

In conclusion this thesis hypothesises that sequential infection with different dengue virus serotypes elicits highly activated, cross-reactive CTL from memory which produce high levels of pro-inflammatory cytokines. Dengue-specific cross-reactive CD4+ T cell populations are also generated from memory and are capable of producing even greater levels of pro-inflammatory cytokines, and perhaps priming other cell populations. These mediators lead to the development of fluid leak and shock. High-avidity CD8+ T cells are subsequently deleted, perhaps as a consequence of activation-induced cell death, and a more beneficial serotype-specific memory CTL pool generated. These observations have significant implications for our understanding of the role of virus-specific CTL in pathogenesis of dengue disease and consequently for the design of a safe, effective vaccine.

ABBREVIATIONS

µg	Microgramme
µM	Micromolar
ADE	Antibody dependent enhancement
APTT	Activated partial thromboplastin time
AST	Aspartate transferase
BCR	B cell receptor
CD	Cluster of differentiation
CSF	Cerebrospinal fluid
CTL	Cytotoxic CD8+ T lymphocyte (unless CD4 clearly indicated)
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing nonintegrin
DEN-1 to -4	Dengue virus serotype 1 to 4
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
DV	Dengue virus
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
GM-CSF	Granulocytes-macrophage colony stimulating factor
Hb	Haemoglobin
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JE	Japanese encephalitis
mg	Millgramme
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
ml	Millilitres
mM	Millimolar
mRNA	messenger Ribonucleic acid
NS	Non-structural protein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pD1 to pD4	Peptides representing variant epitopes from each dengue serotype
PE	Phycoerythrin
pMHC	Peptide complexed with MHC
TNF	Tumour necrosis factor
TCR	T cell receptor

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CHAPTER 1 INTRODUCTION

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are febrile illnesses caused by members of the genus *Flavivirus,* the dengue viruses. There are four distinct serotypes (DEN 1 to 4) of which humans are the primary vertebrate host and *Aedes* mosquitoes (e.g. *A. aegypti*) the primary vector. Most infections are asymptomatic but for those with symptoms clinical manifestations range from a mild fever (dengue fever, DF) to a potentially fatal syndrome of shock characterised by plasma leakage (dengue shock syndrome, DSS) with or without visible haemorrhage (dengue haemorrhagic fever, DHF)(1, 2).

Dengue is not a new disease. The first recorded outbreak of a dengue-like illness was in China in around 992AD(3) and epidemics of an illness compatible with dengue occurred on three continents (Asia, Africa and North America) at the end of the 18th century. However the 20th century has seen dramatic changes in the epidemiology of the disease with a steady increase in the incidence of dengue virus infection throughout the world and the rise of a newly recognised severe form – dengue haemorrhagic fever. Dengue is now a global disease of the tropics with the potential to affect nearly half of the population of the globe(4, 5) (Figure 1) and is considered by many to be among the most significant *emerging* diseases in the world(6). There is no specific therapy and no commercial vaccine available at present.

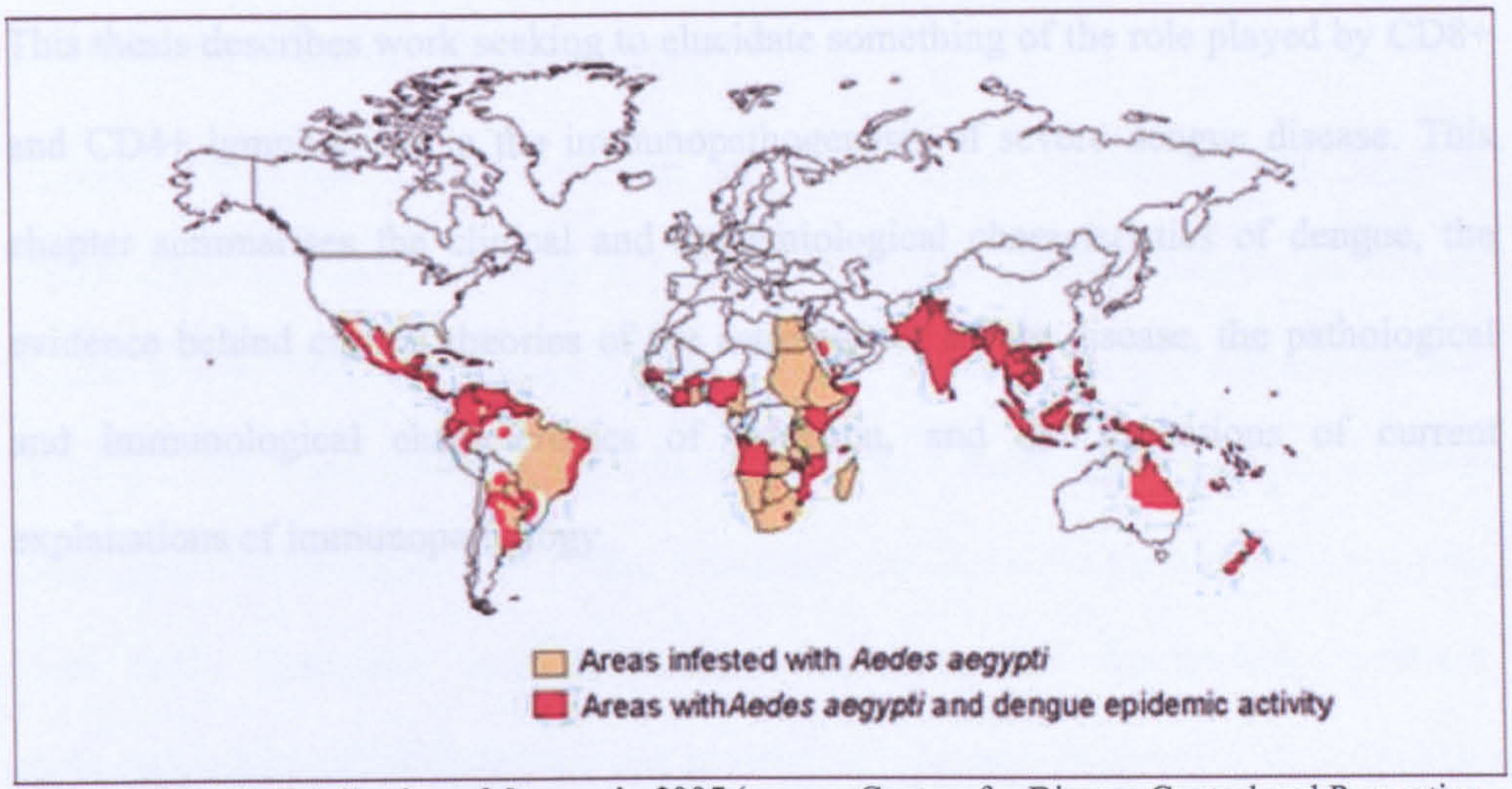


Figure 1. World distribution of dengue in 2005 (source: Centres for Disease Control and Prevention, Atlanta, USA)

Epidemiological studies in the 1970s demonstrated that the severe form of disease, DHF, was seen most frequently in those experiencing secondary infection(7). A process of "antibody dependent enhancement" (ADE) was proposed as the explanation of this link between disease severity and immunological memory(8, 9). This theory suggests that in certain situations pre-existing antibody fails to neutralise circulating virus and instead facilitates its uptake into cells such as macrophages where it is free to replicate. This leads to higher viral loads and more severe disease than that seen in naïve individuals.

ADE can be used to explain many of the clinical and epidemiological observations relating to dengue infection. However its dominant position has led to many overlooking the part that may be played by other components of the immune system – particularly the contribution of cellular immunity.

This thesis describes work seeking to elucidate something of the role played by CD8+ and CD4+ lymphocytes in the immunopathogenesis of severe dengue disease. This chapter summarises the clinical and epidemiological characteristics of dengue, the evidence behind current theories of the aetiology of severe disease, the pathological and immunological characteristics of infection, and the limitations of current explanations of immunopathology.

Clinical features of dengue disease

Dengue virus infection may cause a wide spectrum of illness (Figure 2). Many cases are asymptomatic or result in a mild fever. Young children in particular may experience an undifferentiated fever, often with a maculopapular rash.

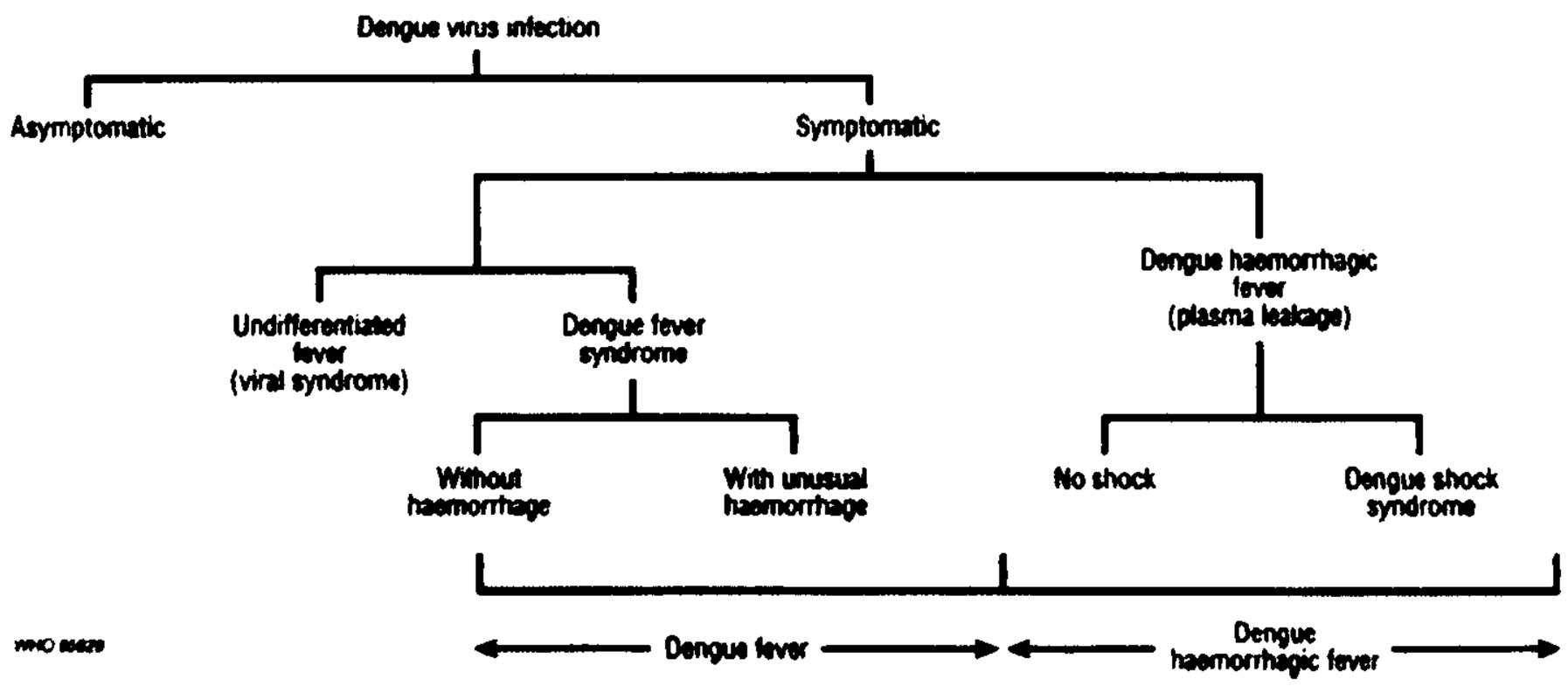


Figure 2. The spectrum of dengue infection. From "Dengue haemorrhagic fever: diagnosis, treatment, prevention and control." 2nd edition. Geneva : World Health Organization.

Clinical features of "dengue fever"

"Classic" DF is seen in older children and adults. It is a non-fatal febrile illness of abrupt onset and lasts 5-7 days. A 5-10 day incubation period follows an infected mosquito bite. Prodromal symptoms of headache, backache, fatigue and malaise may develop a few hours before fever, which is of abrupt onset. Other symptoms include body pain, muscle ache, retro-orbital headache, macular rash, photophobia, eye pain, sore throat, testicular pain, delirium, lympadenopathy (particularly posterior auricular), constipation, diarrhoea and prostration, nausea and vomiting. It is therefore easily clinically confused with other viral or even bacterial infections (e.g. leptospirosis, typhoid). Severe muscle pain and osteoarthralgia are frequently found in adults ("breakbone fever" – a name coined by those caught up in the Philadelphia outbreak of 1780(3)) but less commonly in children. Fever lasts 5-7 days and may return to normal in the middle of the febrile period producing the classic "saddleback" temperature chart. Hepatomegaly has been reported. Some epidemics of DF have been accompanied by features of bleeding (e.g. gingival, petechiae, purpura, epistaxis, menorrhagia, gastrointestinal). Patients with existing peptic ulcer disease may develop severe potentially life threatening bleeding. It is important to note that these cases of DF with unusual bleeding(10) are distinct from DHF/DSS, a syndrome characterised by plasma leakage and haemoconcentration due to increased vascular permeability.

Even in fairly mild cases convalescence may be prolonged with some adult patients experiencing fatigue and depression.

Clinical features of "dengue haemorrhagic fever"

DHF is typically characterised by high fever, haemorrhagic manifestations, hepatomegaly and circulatory failure(11). DHF/DSS resembles DF in the early stages. However at the time of, or shortly after, defervescence circulatory disturbance develops. In mild cases this may manifest as sweating, restlessness and cold peripheries. Patients may recover spontaneously but in severe cases shock rapidly develops with tachycardia and peripheral cyanosis as a consequence of plasma leakage. Peripheral oedema is unusual but pleural effusions and ascites are the norm(12). Laboratory tests may reveal moderate to marked thrombocytopenia, haemoconcentration (elevated haematocrit) and hypoproteinaemia. These changes are usually apparent before the onset of crisis at the end of the febrile phase and facilitate early identification of those patients developing incipient shock(3). The commonest haemorrhagic manifestations are a positive tourniquet test (Figure 3), easy bruising and bleeding at sites of venepuncture. Recent studies have however demonstrated that the tourniquet test is poorly sensitive for dengue and does not reliably distinguish between DHF and DF(13). The liver is usually palpable early in the febrile phase and hepatomegaly is observed more frequently in shock than non-shock cases. Jaundice is not usually seen. Patients who go on to develop shock may develop acute abdominal pain shortly beforehand. Without intervention the course of shock is short but life threatening with death occurring as little as 12 hours after onset. Prolonged shock may be associated with metabolic acidosis and disseminated intravascular coagulation (DIC) with gastrointestinal haemorrhage and rarely intracranial bleeds. The period of plasma leakage rarely lasts longer than 48 hours and if managed quickly and appropriately with fluid replacement recovery is rapid with the course of uncomplicated disease being around 7-10 days.

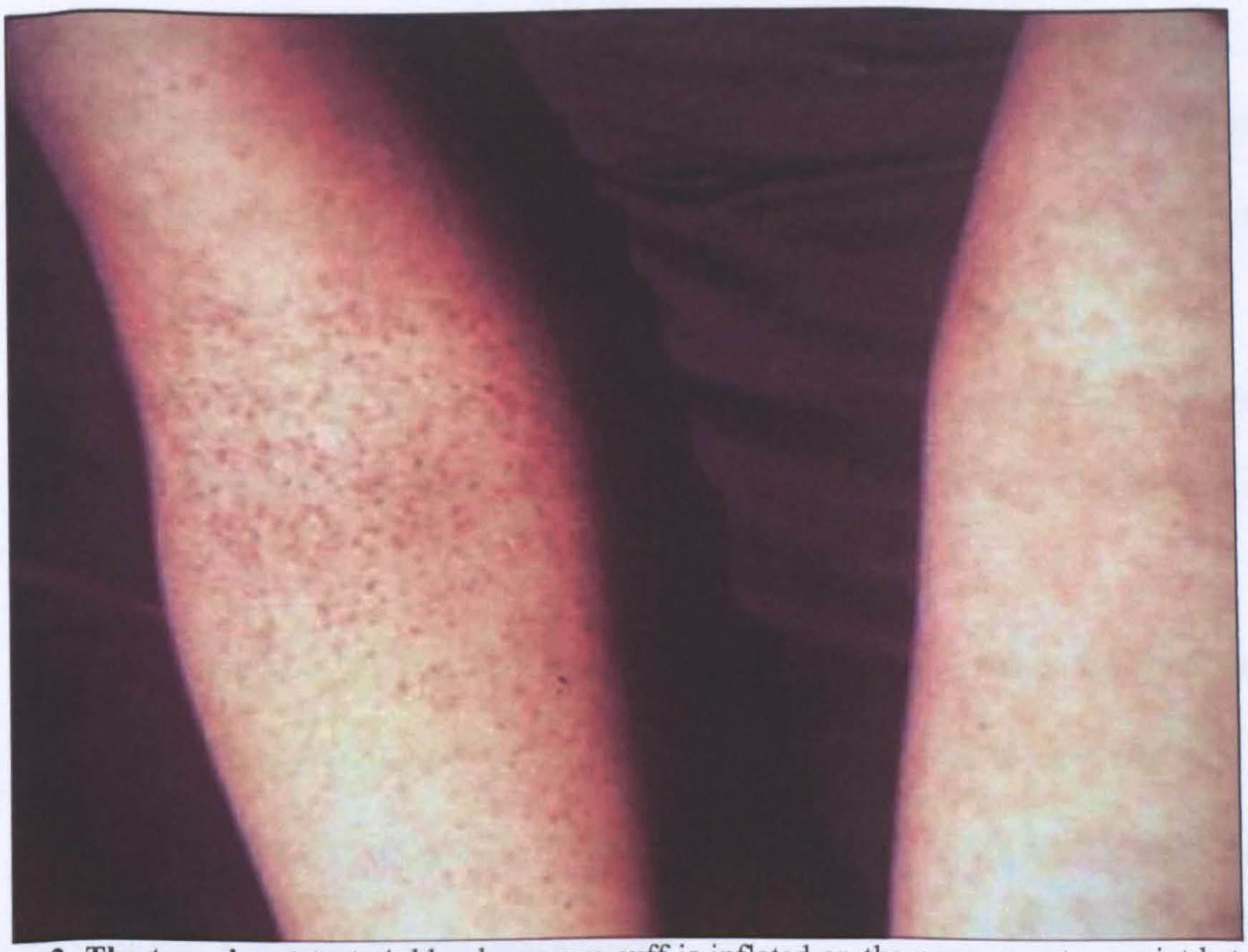


Figure 3. The tourniquet test. A blood pressure cuff is inflated on the upper arm to a point between the diastolic and systolic pressures for 5 minutes. The test is considered positive when there are 20 or more petechiae per 1 inch square of skin observed. It can be negative if the patient is in profound shock and only become positive after recovery from shock.

Complications of dengue

- Central nervous system – febrile convulsions, spasticity, paresis, changes in consciousness, encephalopathy, encephalitis, intracranial bleeds, cerebral oedema.
- Iatrogenic – fluid overload and respiratory or heart failure, sepsis, pneumonia.
- Liver failure – either primary viral cause or secondary to circulatory failure(3).

Laboratory findings (14)

- *Thrombocytopenia* – often seen in DF and invariably seen in DHF. Platelets usually drop below 100 000 per mm^3 between day 3-8 of illness.

- *Haematocrit* – rises in all cases but more pronounced in those with shock. An increase of 20% or more is considered confirmation of plasma leakage. It may be affected by early fluid replacement or bleeding. Both the fall in platelets and rise in haematocrit occur before defervescence and the onset of shock.
- *White cell count* – may be low or mildly raised. A relative lymphocytosis may be observed shortly before defervescence. Neutrophils usually fall towards the end of illness.
- *Clotting screen* – prothrombin time (PT) and activated partial thromboplastin time (APTT) are increased in up to 50% of patients and certain clotting and fibrinolytic factors may be decreased, particularly in those severe cases developing a degree of liver dysfunction.
- *X-ray* – pleural effusion (usually right sided) – the extent correlates with disease severity (Figure 4). Bilateral effusions are common finding in shock.

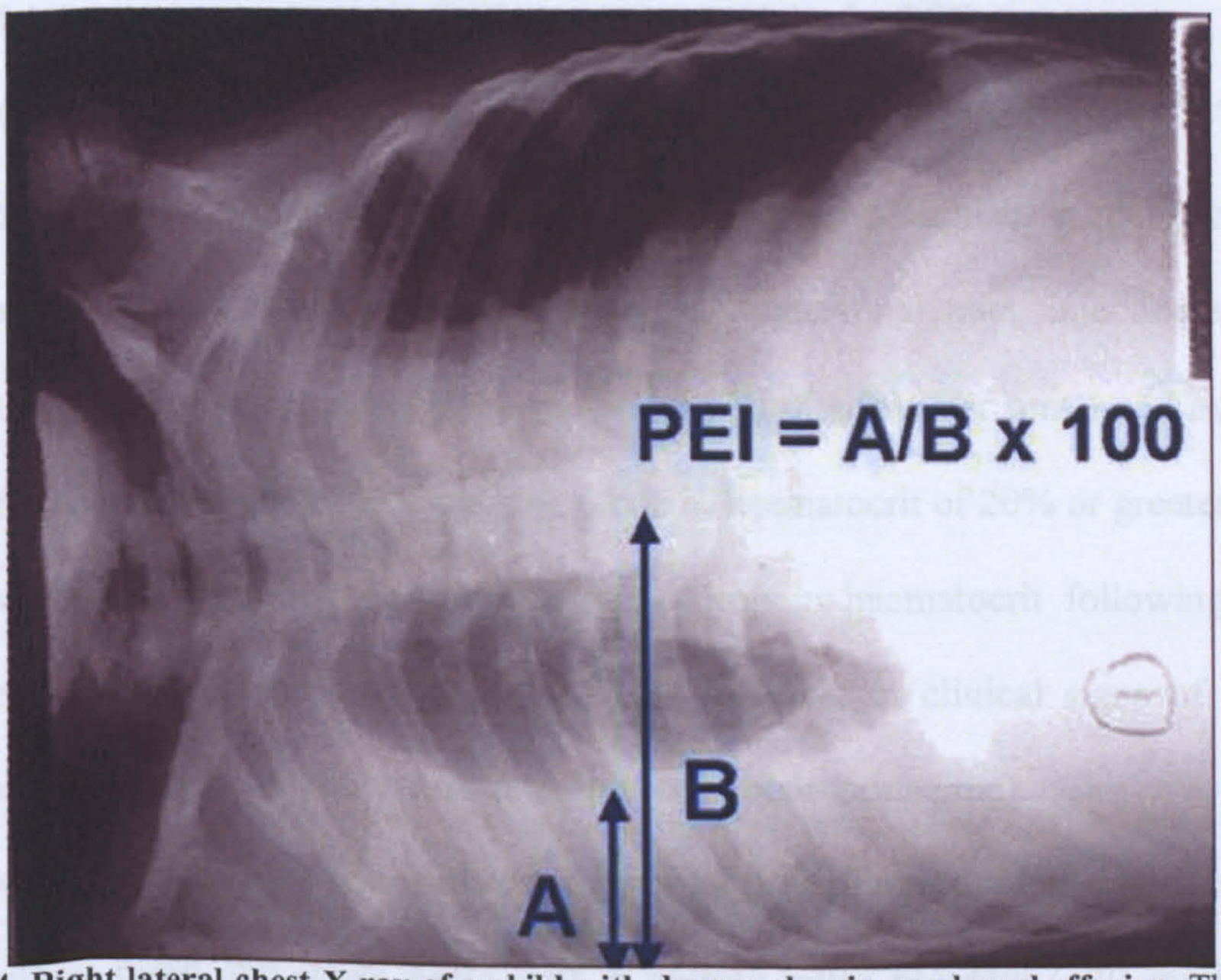


Figure 4. Right lateral chest X-ray of a child with dengue showing a pleural effusion. The extent of the effusion (in this case quantified by the pleural effusion index, PEI) correlates with disease severity.

- *Other*: albuminuria, hypoproteinaemia, liver function test changes, metabolic acidosis.

The World Health Organisation clinical classification of dengue

The World Health Organisation has produced case definitions for clinical dengue disease (14).

- *Probable dengue fever* – 2 or more of headache, retro-orbital pain, myalgia, arthralgia, rash, haemorrhagic manifestations, leucopenia AND either occurrence at the same time and place as other confirmed cases OR supportive serology.
- *Confirmed dengue fever* – a case confirmed by laboratory criteria including: isolation of dengue virus from serum or autopsy samples; demonstration of viral antigen from serum, CSF or tissue samples; demonstration of fourfold or greater change in reciprocal IgG or IgM antibody titres in paired samples; or detection of dengue virus genome in CSF or autopsy samples by PCR.
- *Dengue haemorrhagic fever* – all of: fever or history of fever lasting 2-7 days, haemorrhagic tendencies (at least one of bruising, petechiae, purpura, positive tourniquet test, bleeding from mucosa, gastrointestinal tract, injection sites or other locations), thrombocytopenia (<100 000 platelets per mm^3) and evidence of plasma leakage (as indicated by: a rise in haematocrit of 20% or greater above average for age, sex and population; a drop in haematocrit following fluid-replacement greater or equal to 20% of baseline; or clinical signs of plasma leakage such as pleural effusion, ascites or hypoproteinaemia).
- *Dengue shock syndrome* – all of the criteria for DHF plus evidence of circulatory failure: rapid and weak pulse with a narrow pulse pressure (<20 mmHg), or hypotension for age with cold, clammy skin and restlessness.

There are four grades of severity of DHF (Figure 5). Grades III and IV are considered to be DSS.

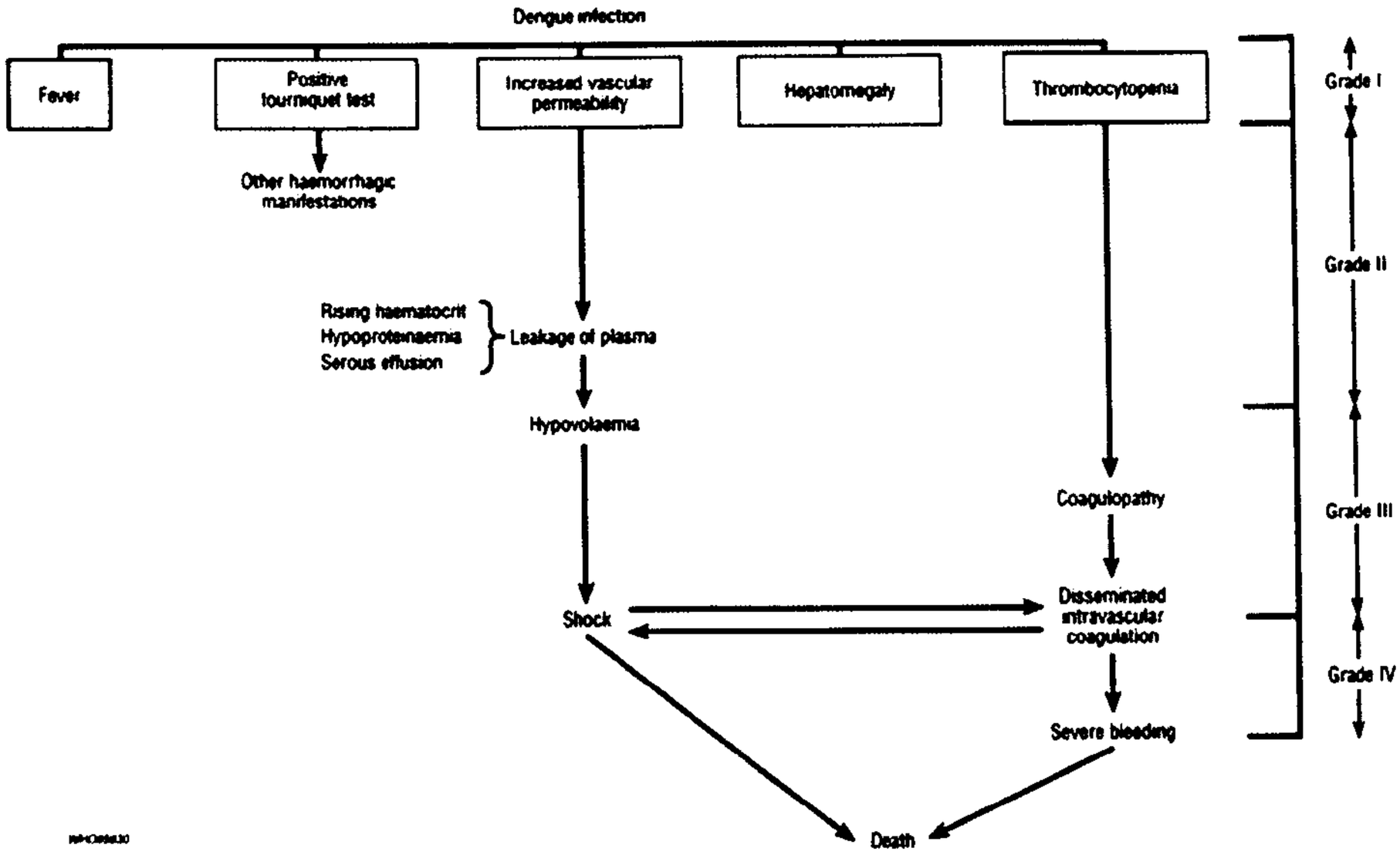


Figure 5. The clinical spectrum of dengue haemorrhagic fever. From "Dengue haemorrhagic fever: diagnosis, treatment, prevention and control." 2nd edition. Geneva: World Health Organization.

- Grade I – fever with non-specific symptoms and the only haemorrhagic manifestation a positive tourniquet test or easy bruising.
- Grade II – the above plus spontaneous bleeding (usually skin haemorrhages).
- Grade III – circulatory failure with rapid, weak pulse and narrow pulse pressure with hypotension, cold clammy skin and restlessness.
- Grade IV – profound shock with an undetectable blood pressure or pulse.

The WHO system of classifying dengue disease together with the implementation of fluid management guidelines (see below) saw a dramatic fall in the case fatality rate of severe dengue(15, 16). However not all cases fit neatly to the criteria described and

new terms such as "dengue fever with unusual haemorrhage" and "dengue with signs associated with shock" have been introduced. In some outbreaks bleeding and thrombocytopenia have been as common in the dengue fever classified patients as those considered to have DHF(17). It may be that dengue disease severity exists as a continuum rather than distinct clinical entities. This has led some to propose the development of new systems of classification(16).

Diagnosis

By the time an individual infected with dengue develops symptoms the virus is widely disseminated and may be identified in serum, circulating blood cells and the tissues of the immune system. Peripheral blood mononuclear cells are infected within a few days of the mosquito bite and anti-dengue antibodies arise within a few days of symptoms. Virus remains detectable for roughly the period of the fever. Diagnosis by viral recovery or detection is preferable but serological techniques are used to confirm most dengue infections. Single specimens can in most cases allow a presumptive diagnosis of *recent* infection, but a conclusive diagnosis of *acute* infection can only be made when rising levels of anti-dengue immunoglobulin are detected in paired sera.

Serological diagnosis

The antibody response

Flavivirus naïve individuals (including those not immunised against yellow fever) mount a primary type response, producing IgM. This is detectable in 50% of patients whilst still febrile and within 3 days of defervescence in the remainder(18). IgM titres peak at 2 weeks after symptom onset, becoming undetectable by 3 months. IgG is

present in only low levels in the febrile or early convalescent phase, arising shortly after IgM. Patients experiencing primary infection may mount an antibody response cross-reactive between dengue serotypes early in disease. By months 3-6 a monotypic antibody response specific for the infecting serotype develops (Figure 6).

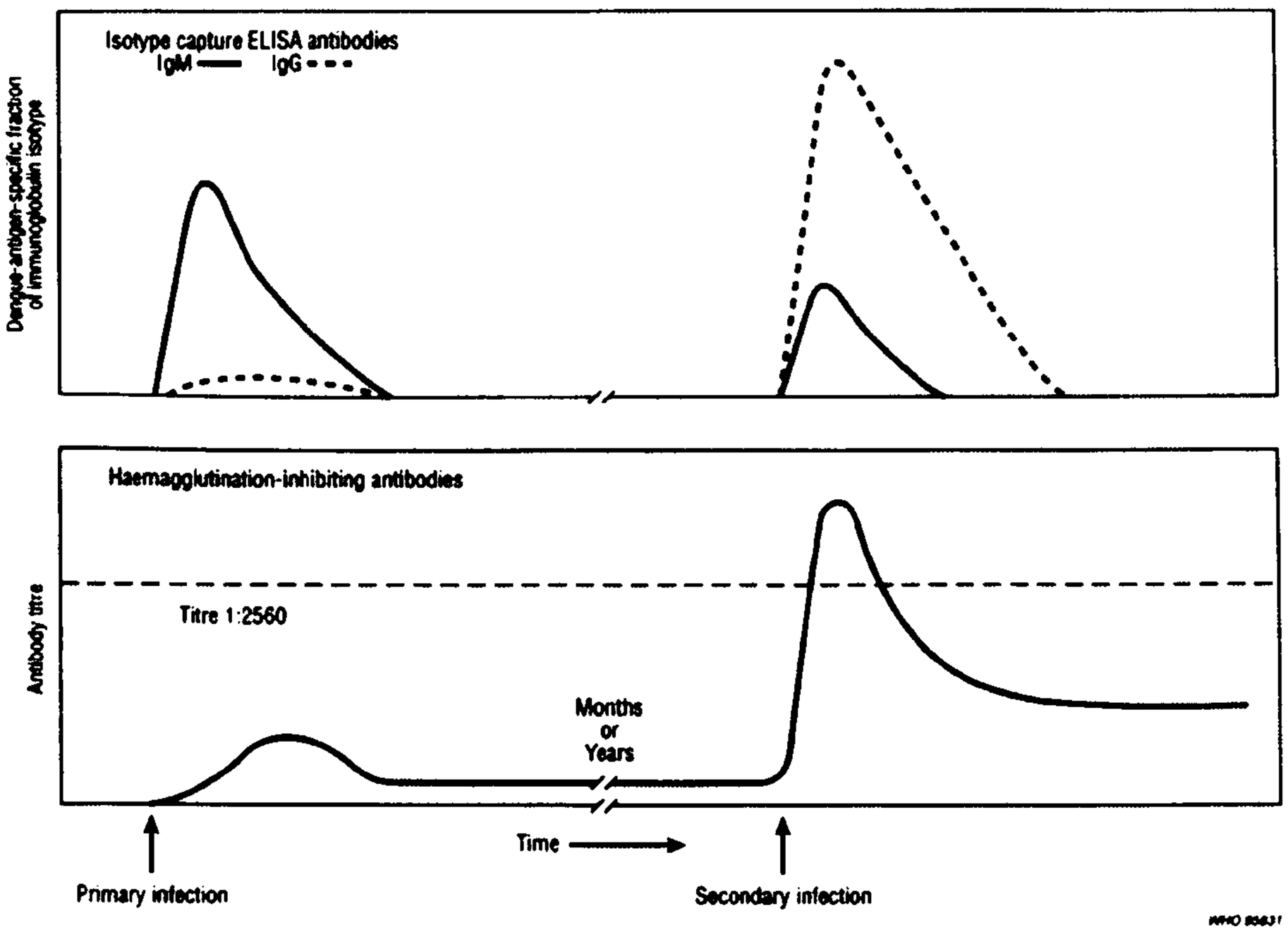


Figure 6. Primary and secondary immunological responses to dengue infection. From Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 1997 World Health Organization, Geneva, Switzerland.

Despite the antigenically related nature of the dengue viruses two or more viral types can sequentially infect a host – this is of great significance clinically and epidemiologically and is discussed in detail below. The antibody response to a sequential infection is very different from that elicited by the primary infection. This sequential response may occur when the host has experienced infection with a non-dengue flavivirus – at least half of which infect humans. Those patients mounting a non-naïve, secondary antibody response to dengue infection produce predominantly

IgG. IgM is produced in a similar manner to that seen in primary infection but at much lower levels and in parallel with the high levels of IgG. Both titres peak at about 2 weeks after symptom onset, IgG declining over the next 3-6 months and IgM remaining detectable in only 30% at 2 months. The IgG is broadly cross-reactive – this makes it harder to identify the infecting viral serotype and it is difficult to recover virus from serum samples taken after defervescence (see below). In some cases IgM is not detected. Its presence or absence and titre may be related to the antigenic relatedness of the sequentially infecting virus – a closely related dengue serotype may not present many new epitopes, unlike a distantly related flavivirus.

The duration of humoral immunity protecting against clinically overt homotypic dengue infection appears long. Many people living through a big epidemic of DEN-1 and DEN-2 in Athens in the 1920s were found still to have neutralising antibodies to the infecting serotype 40 years later(19). Similar observations have been made in Japan and from those undergoing experimental inoculations(20).

Serological tests

Serological diagnosis using paired samples (at least 10 days apart) is the most widely used means of confirming dengue infection due the simple techniques involved and widespread availability of reagents. False positive results can occur and may be due to cross-reactive antibodies (either between dengue serotypes or other flaviviruses) and the phenomenon of "original antigenic sin" (B-cell clones producing antibody to the original flavivirus infection are restimulated and produce early antibody that has a greater affinity for that first infection than the current virus(21)). Detection of anti-

dengue IgM alone may allow the diagnosis of dengue to be confirmed but not allow identification of the specific serotype.

The different techniques of antibody identification vary in what they detect and their cross-reactivity between dengue serotypes and other flaviviruses. They are briefly summarised below. The most widely used are IgM antibody capture-ELISA and Haemagglutination Inhibition.

- *Antibody detection by ELISA* – MAC-ELISA (IgM antibody capture-ELISA) can measure a rise in dengue specific IgM even 1-2 days into the acute phase of illness in both primary and secondary infection. In cases where only a single specimen is available detection of anti-dengue IgM permits a diagnosis of recent dengue. Negative tests taken before the 6th day of illness should be repeated. A fraction of secondary dengue cases will have a low or negative IgM reaction and IgG testing may be indicated. Anti-flavivirus IgM is complex-specific and allows differentiation of dengue from other flaviviral infections. Some cross-reactivity does occur however. The test can also be used to detect IgM in the CSF – IgM does not normally cross the blood-brain barrier and its presence implies viral replication within the CNS. The assay is particular useful for laboratories performing a high volume of testing. Anti-human IgM antibody is bound to a plate and used to non-specifically capture IgM from serum samples. The captured antibody is then reacted with dengue antigens (either separately, or all 4 in a pool since the test is not reliably serotype specific). A signal generating system (e.g. anti-dengue antibody conjugated to horseradish peroxidise) then detects the presence of bound antigen. IgG-ELISA tests exist but are not very

specific cross-reacting with other flaviviruses and unable to differentiate between dengue serotypes. They are however well suited to the analysis of a large number of samples (useful in seroepidemiological studies(22)), are as sensitive as haemagglutination inhibition based assays and can be used to differentiate primary from secondary infections. They may not correlate well with HI in primary infection because the HI test measures both IgM and IgG.

- *Haemagglutination-inhibition (HI) test* – simple and reproducible, the strength of this test is that it uses agents that can be prepared locally. It does however require paired sera separated by more than 7 days and does not distinguish between closely related flaviviruses (e.g. dengue, JE and West Nile). Dengue viruses agglutinate gander erythrocytes as well as trypsinised type O human red blood cells. The HI test relies on the ability of dengue antibodies to inhibit this agglutination. Sera are extracted with kaolin or acetone (to remove non-specific inhibitors of agglutination) and absorbed with gander or trypsinised type O human red cells. All sera from a single patient are titrated and tested in the same assay. The endpoint of the titration is the highest dilution of serum that inhibits agglutination of a standard amount of antigen (usually 4-8 haemagglutinating units of the four dengue antigens). A fourfold or greater change in HI titre between paired sera is considered diagnostic for recent infection. The test measures all classes of antibody. In primary infections detectable HI antibody appears after day 5 and rises over weeks, rarely exceeding 1:640. Secondary or tertiary infections see a rapid elevation of antibody within a few days – acute samples are often HI positive. Titres of 1:20480 are not uncommon in convalescence and may persist for several weeks(3, 14). HI antibodies are cross-reactive even in primary dengue infections – the test can be positive using an

antigen from almost any flavivirus. The HI titre falls over years and absence of HI antibodies does not equate with immunological naivety.

- *Plaque reduction neutralisation test* – this is the most type specific of the traditional serological techniques and is considered the best measure of true immunity after vaccination. It requires carefully titrated virus stocks and tissue culture facilities. Dilutions of heat-inactivated serum are incubated with defined amounts of virus. The non-neutralised viral remnant is adsorbed onto a monolayer of susceptible cells and the resulting plaques counted. The endpoint of the titration is the highest dilution of serum that reduces the number of plaques by 50-90%. A fourfold or greater rise in titre between acute and convalescent samples is diagnostic of a current infection. Relatively type-specific neutralising antibodies are present in early convalescence following primary infection. Specificity improves a few months after infection. After secondary infection high titre neutralising antibody is produced against at least 2, and usually all 4 dengue virus serotypes and often other flaviviruses(23, 24). Testing of appropriately timed samples from subjects who have undergone various combinations of sequential infections reveals that the highest neutralising antibody titre in convalescent serum is directed against the virus with which the patients was *previously* (not most recently) infected. This observation led to the theory of original antigenic sin in dengue(21).
- *Complement fixation* – technically difficult and the least sensitive, complement fixation is not widely performed. Complement-fixing antibody generally appears later than IgM or HI and is usually more specific (although not so specific as neutralising assays) so may be useful in confirming dengue infection in patients with serum samples taken late in the course of infection.

Viral detection

Definitive diagnosis is made by the detection of virus in culture. The period during which this can be achieved is short and viral culture is rarely successfully achieved from samples taken at the time of, or shortly after defervescence – the presence of newly generated dengue antibody interferes with viral culture. Identification of dengue RNA by reverse-transcription polymerase chain reaction (PCR) is fast but technically complicated and in the absence of good technique and proper precautions contamination can lead to false positive results. Dengue RNA or antigen can be identified in individual cells by *in-situ* hybridisation or immunocytochemistry

- *Viral isolation* – samples should be taken during the first 5 days of illness. The most sensitive culture technique is the inoculation of material into adult or larval mosquitoes. Infection is then detected by immunofluorescence of a tissue smear produced from the crushed mosquito head. Maintaining mosquito colonies is a considerable investment of time and resources. Mosquito cell lines, although less sensitive, are much more convenient. The presence of cytopathic effect cannot be relied upon as this is not produced by every viral strain – immunofluorescence should be performed. The least sensitive means of culture is in vertebrate cell lines, or intracerebrally inoculated mice.

- *Antigen detection in fixed tissue* – flavivirus antigen can be detected in PBMC from patients with dengue, most reliably during the febrile phase of illness, as well as in the liver, lung, thymus, lymph nodes, spleen and bone marrow of post mortem specimens. Fluorescent antibody and avidin-biotin enzyme assay are available for the visualisation of viral antigen in fixed tissues.

- *Reverse transcription-PCR detection of dengue RNA* – dengue-specific oligonucleotide primers allow PCR-based detection of dengue virus even during convalescence when circulating antibodies would otherwise preclude its detection. They are prone to false positive results due to contamination.

Management

The prognosis of DHF depends on early diagnosis and recognition of plasma leakage. Frequent monitoring of platelet count and haematocrit can allow leakage to be detected in its earliest stages, usually at around the 3rd day of illness. Treatment is aimed at fluid replacement to maintain the circulatory volume for the 24-48 hours that increased vascular permeability persists. Intravenous fluids should be isotonic. Recent clinical trials have shown no significant difference in outcome between patients managed with colloids, Ringer's lactate and normal saline although those with severe shock managed with Ringer's lactate took longer to recover(15, 16). Mild cases of DHF do not necessarily need hospitalisation. Cases with low platelet counts, high haematocrit, haemorrhage other than mild petechiae or any clinical signs of shock should be managed as inpatients. DF, DHF I and II are managed with antipyretics (e.g. paracetamol. Aspirin is contraindicated – it may exacerbate haemorrhage and is associated with Reye's syndrome in children) and simple oral hydration. A haematocrit rise of 20% or more indicates the need for IV fluid therapy. Fluids should be given judiciously, reassessing the requirements for the next few hours at regular intervals as guided by cardiovascular clinical signs and changes in haematocrit(3, 14). Excessive volume replacement risks pleural effusion, respiratory distress and ascites during the convalescent phase when fluid is reabsorbed. Other treatments should be

given as indicated, e.g. blood transfusion, correction of electrolyte and metabolic disturbance, management of clotting abnormalities, liver impairment etc..

The aetiology of DHF

DHF is a relatively new disease. Cases were described at the start of the 20th century and the first modern outbreaks reported in Thailand in the 1950s. The post-war years saw a rapid expansion in the frequency and geographical spread of DHF outbreaks(3). The reasons behind this rapid expansion are discussed further later – here we consider the aetiology of severe dengue disease.

Previous infection

DHF has been most studied where it first appeared – in Southeast Asia – where it is predominantly a disease of childhood. In the tropical Americas it is seen in all age groups(25). Epidemiological studies of Asian epidemics have repeatedly demonstrated two key observations(7, 26):

1. Such epidemics take place in regions where multiple types of dengue virus serotypes are simultaneously, or sequentially endemic.
2. Patients meeting the clinical criteria for DHF have secondary dengue antibody responses, unless less than 1 year of age in which case they exhibit primary responses.

Prospective sero-epidemiological studies have confirmed these observations – virtually all DHF cases occur in those experiencing a secondary infection(27, 28).

Thus a key, perhaps *the* key characteristic of those patients developing severe dengue disease is evidence of previous infection. It would appear that infection with one serotype does not provide absolute protection against later infection with another, and may in fact lead to more severe clinical disease. Perhaps the strongest evidence to support the sequential infection hypothesis is to be found in Cuba which experienced a DEN-1 outbreak in 1977, followed by a DEN-2 outbreak in 1981. This second outbreak saw a large number of DHF cases in all but those aged between 1 and 3 years old – those who were too young to have previously been infected with dengue and too old to have residual maternal antibody(29). This is another fascinating epidemiological observation. Severe disease in infants is seen in children less than 1 year of age born to dengue immune mothers(30). The vast majority of women in endemic areas of child-bearing age have antibody to dengue virus(31). These maternal antibodies effectively neutralise all serotypes of dengue. As might be predicted transplacental transfer of maternal antibody protects infants from dengue infection in the early months of life – infants less than 3 months of age are rarely hospitalised for dengue virus infection(32). Studies in Thailand demonstrated that the rates of hospitalisation peaked at an age of 7 to 8 months when they were up to 8 times that of a 1 to 3 month old and twice that of a 3 year old. Maternal antibody to dengue declines at a constant rate with a half life of around 35 days. Thus babies born to dengue immune mothers are initially protected from severe infection but once maternally acquired antibody falls below the protective level it appears that there is a time window during which infection is enhanced. The age at which an infant develops DHF is related to the level of antibody acquired from the mother(30). Eventually maternal antibody disappears and if the infant remains dengue naïve their risk of severe infection returns to that of an individual experiencing primary infection.

Several mechanisms have been proposed as the means by which previous infection and severe disease are linked. The pre-eminent of these is that of antibody-dependent enhancement (ADE). ADE is a neat explanation of both why severe disease is seen in infants born to dengue-immune mothers from around 6 until 9 months of age, and why most other cases occur in those experiencing secondary infection. It appears that antibody generated after a primary infection is protective against reinfection by all dengue serotypes for a relatively short period of time. As antibody levels wane so does protection against heterotypic virus and infection is instead enhanced. Enhancing antibody might act by increasing the replication of the virus through facilitating uptake into its target cell, or by altering the tissue tropism of the virus. Interestingly enhancement of dengue infection of cell lines is seen *in vitro* with antibodies to flaviviruses other than just dengue – no epidemiological association has yet been made with severe disease and previous exposure to such viruses. The experimental evidence for ADE is discussed further below.

Viral strains

Co-circulation of viral serotypes appears to be necessary but not sufficient for DHF. DHF and DSS with fatalities have been documented in adults and children with primary dengue infection(33). During the 1970s and 80s three or four serotypes were present in tropical regions of the Americas and the Indian subcontinent either simultaneously or sequentially with no epidemic DHF. It is likely that viral factors influence pathogenicity and certainly viruses differ genotypically and phenotypically in ways that affect their virulence in experimental systems. Laboratory strains of dengue virus can be modified by propagation, changing their tissue tropism,

temperature sensitivity and cytopathic potential. However it is less easy to prove that natural dengue virus strains differ in virulence. Secondary infections caused by dengue viruses of Asian origin do seem to have an association with more severe disease(34). There were a large number of cases of DHF in the Americas during an outbreak of DEN-2 which was more closely related to Asian strains than previously circulating American viruses(35). Similar observations were later made with the introduction of DEN-3 virus into the Americas(36). Outbreaks of DEN-3 have been recorded in endemic areas among immune populations which were associated with low viraemia and caused only mild disease(37). Yet separating the effects of the introduction of a specific strain from more general changes in epidemiology (e.g. the more widespread circulation of multiple viral serotypes and increased rate of secondary infection) is not straightforward. DEN-2 virus strains associated with DHF have been shown to have different growth and enhancement characteristics(38, 39). Similar observations have not been made with other dengue serotypes.

Age

DHF/DSS tends to affect the young and the elderly. In the Cuban outbreak of 1981 everyone from age 2-40 had the same exposure history yet hospitalised patients peaked at 8-11 years of age and fell to baseline among the mid-teens and above(40). Few severely ill adults have severe vascular leakage – it is possible that the capillaries of young children are more prone to cytokine-mediated permeability changes than those of adults(41) (Figure 7).

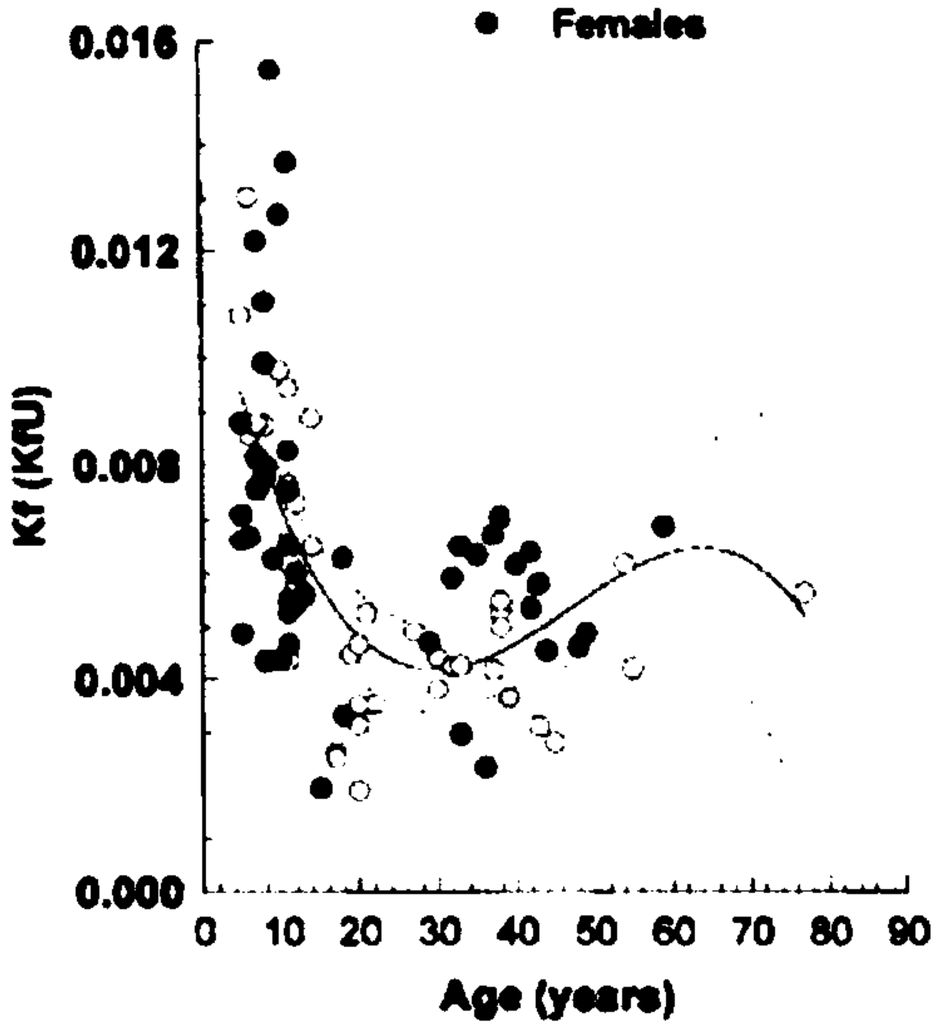


Figure 7. The Kf data obtained from 89 healthy Vietnamese volunteers aged 5 to 77 years. Kf is a measure of vascular permeability derived in this case from changes in tissue volume as a result of increasing the pressure in a proximally placed cuff. Filled circles represent women, empty circles men. The solid line represents a third-order polynomial fit for these data and the dotted lines the 95% confidence limits for that fit. From Gamble, J., D. Bethell, N.P. Day, P.P. Loc, N.H. Phu, I.B. Gartside, J.F. Farrar, and N.J. White. 2000. Age-related changes in microvascular permeability: a significant factor in the susceptibility of children to shock? Clin Sci (Lond) 98:211-216.

General health

Undernourished infants are at lower risk of DHF than those with a good nutritional status(42) – perhaps as a consequence of the suppressed cellular immune response seen among those with malnutrition(43, 44). Individuals with peptic ulcer disease are more likely to experience severe bleeds in the course of dengue infection.

Host genetics

Host factors play a part in the development of severe disease. There is significant observational evidence that black people have a lower risk of developing DHF/DSS than white people despite similar levels of infection – for example despite hyperendemic transmission in Haiti cases of DHF are extremely rare(45, 46). It has

been suggested that the millennia over which yellow fever has been enzootic in Africa has resulted in selection for resistance genes that may protect against severe flavivirus disease(3). Increased expression of HLA molecules on the surface of infected cells has been described during flavivirus infection(47) and it has been suggested that this could contribute to immunopathogenesis. A number of HLA alleles have been found to have statistical association with protection from (A*0203, A*29, A*33, B*52 DRB1*04) or a predisposition to (A*1, A*0207, A*24, B*51, DQ1) the development of DHF(48). Other genes noted to have an association with DHF include the vitamin D receptor, FcγR II(49) and certain TNF-α polymorphisms(50).

The rise of dengue

The epidemiology of dengue has changed dramatically over the last 100 years. To understand why this is it is useful to briefly consider the evolution and the geographical of spread of both the virus and its vectors.

The origin of the viruses

It is thought that the viruses originated in a forest cycle involving primates and canopy dwelling mosquitoes (51). Such cycles have been identified in both Southeast Asia and Africa and it is likely that dengue was originally a monkey virus with cross-species transmission to humans occurring independently with each of the four serotypes(52). Studies of nucleotide substitution rates have estimated that the emergence of dengue as a distinct virus took place around 1000 years ago(53), corresponding approximately with the earliest reports of dengue-like illness. These outbreaks probably occurred sporadically as a result of intermittent cross-species

transmission from sylvatic cycles – in much the same way a yellow fever behaves today. Astonishingly it appears that cross-species transmission from monkeys to humans with the establishment of a sustained human/mosquito cycle occurred between 320 (DEN-2) and 125 (DEN-1) years ago, facilitated perhaps by the social and environmental changes associated with urbanisation and trade that provided the virus access to a large pool of susceptible people. The four serotypes are phylogenetically distinct, often to the same degree as other "unrelated" flaviviruses(54). Most genetic diversity currently seen within each dengue serotype is estimated to have appeared almost simultaneously and only during the past century. This makes dengue only a little older than HIV, which is thought to have arisen in human populations around 70 years ago(55). It is not known how the virus developed into four serotypes. It may be they evolved separately in distinct geographical regions. Others suggest they developed within one region, the phenomenon of antibody dependent enhancement promoting the generation of serotype diversity by facilitating infection by related serotypes. However the balance of opinion currently rests with independent evolution. It is likely that the small changes that would have been seen in early divergence would not have been sufficient to avoid complete cross-protection. The phenomenon of antibody-dependent enhancement is more likely to be the result of recent contact between four viruses that have evolved in isolation and by chance have a level of antigenic dissimilarity that allows immune enhancement(52). Whilst only DEN-2 has been documented in Africa all four serotypes have been identified in Asian forest cycles and it is likely that the viruses are of Asian origin.

The vectors

All known vectors of the dengue viruses are mosquitoes belonging to the genus *Aedes*.

In contrast to the virus itself the principal modern vector, *Aedes aegypti* (Figure 8) is thought to have arisen in Africa and travelled to the New World and Asia with the slave trade and commerce of the 17th and 18th centuries. Other species capable of transmission include *A. albopictus* and *A. polynesiensis* and additional species are likely to play a role in certain restricted geographical areas(3). The success of *A. aegypti* as a vector rests on its adapting to the environments created by man. The adults are anthropophilic (feed on humans) and lay their eggs in artificial water containers (abundant in villages and cities). They are dispersed easily by human transport networks. From an African origin in the 17th century they have spread to the Americas, the Mediterranean basin, Asia and the Pacific Islands. Different populations vary in their vector competence. Unlike the anopheline mosquitoes responsible for transmitting malaria they are day biters with feeding times peaking in the mid-morning and late afternoon. They are easily interrupted in their feeding and a single mosquito can infect several members of a household in a short space of time. Infection rates are higher among adult women and pre-school children than adult men, reflecting the increased risk among those staying at home during daylight hours(56). Outbreaks have occurred at schools and hospitals as a consequence of similar day time transmission. Mosquito survival is longer during the rainy season due to high humidity although in some parts of the world and certain urban environments alternative water sources provide ample breeding areas during the dry season. Higher temperatures reduce the extrinsic incubation period, ingested virus reaching the

mosquito's salivary glands more quickly and can facilitate epidemic transmission. Vertical transmission of virus to mosquitoes does occur but at relatively low rates(57).

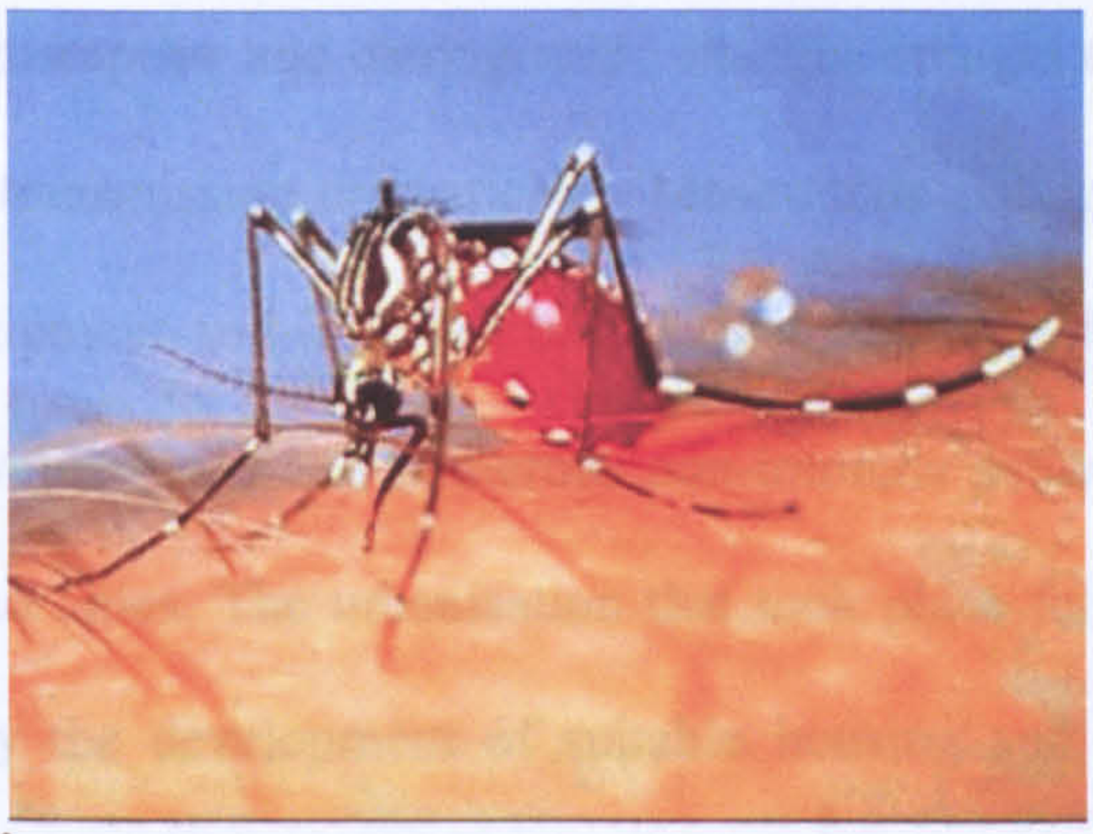


Figure 8. Aedes aegypti

Contemporary epidemiology

Armed with these insights it is easier to understand something of the process behind the dramatic geographical spread of dengue across tropical regions of the world. With the development of cities and clearing of forests dengue viruses moved from their sylvatic cycles into rural environments and later towns and cities spread by *A. albopictus* and other peridomestic species. *Aedes aegypti* had become widespread in tropical cities across the world and the introduction of dengue viruses into these mosquito populations resulted in the major dengue pandemics of the 18th, 19th and 20th centuries. *A. aegypti* became highly adapted to humans and urban life, laying its eggs in the plentiful open water sources and preferring to feed on humans. Its tendency to feed in the day, and on multiple members of a household, made it a highly efficient vehicle for the virus.

Severe and fatal disease associated with dengue was reported sporadically from 1780 but epidemic occurrences were rare and the emergence of severe dengue as a significant public health problem is a phenomenon of the 20th century. In Southeast Asia the ecological disruption and demographic changes brought about by World War 2 increased dengue transmission dramatically. Mosquitoes and eggs were transported to new regions, equipment and junk left behind provided plentiful water collections that made ideal larval habitats and numerous non-immune soldiers moved the virus into new regions and cities. The urbanisation that took place following the war was not accompanied by the development of suitable housing and sanitation and both mosquito and virus thrived. Hyperendemic transmission of multiple virus serotypes – a relatively new phenomenon – was established in most cities of Southeast Asia leading to the first recorded epidemics of DHF in Manila (1953/54), and Bangkok (1968) although sporadic cases had probably been occurring throughout the 1950s. DHF is now a leading cause of hospitalisation and death among children in many countries of Asia.

Despite epidemic dengue in the Caribbean basin during WW2 there were no recorded epidemics from the end of the war until 1963 despite the presence of DEN-2. This was probably due to the *A. aegypti* eradication programmes of the 1940s and 50s aimed primarily at the prevention of urban yellow fever. These were highly successful and many countries completely eradicated the mosquito. However its cessation in the 1970s led to the mosquito's return from those areas in which it survived (Figure 9). Reinfestation left Bermuda and Chile the only previously infested countries free of the mosquito. This period coincided with movement of dengue viruses both within the

continent and the introduction of new serotypes to the continent. Prior to 1977 only DEN-2 and DEN-3 had been identified and each was confined to its own geographical area. The introduction of DEN-1 saw epidemics in Jamaica and Cuba and it then spread throughout the region causing outbreaks of DF. DEN-4 arrived in 1981 and similarly spread, this time with sporadic cases of DSS/DHF. At the same time a new strain of DEN-2 was imported to Cuba from Asia (probably Viet Nam). Unlike the recent DEN-1 and DEN-4 outbreaks this was associated with thousands of cases of DSS/DHF with a mercifully low fatality rate due to the hospitalisation and aggressive fluid management patients underwent. Thus by the end of the 1980s most countries that had previously been dengue-free or had only one serotype circulating became hyperendemic with multiple serotypes present, reporting regular outbreaks of DHF(58). In contrast epidemic dengue tends to occur in populations with little or no immunity – outbreaks can be explosive with attack rates as high as 80%.

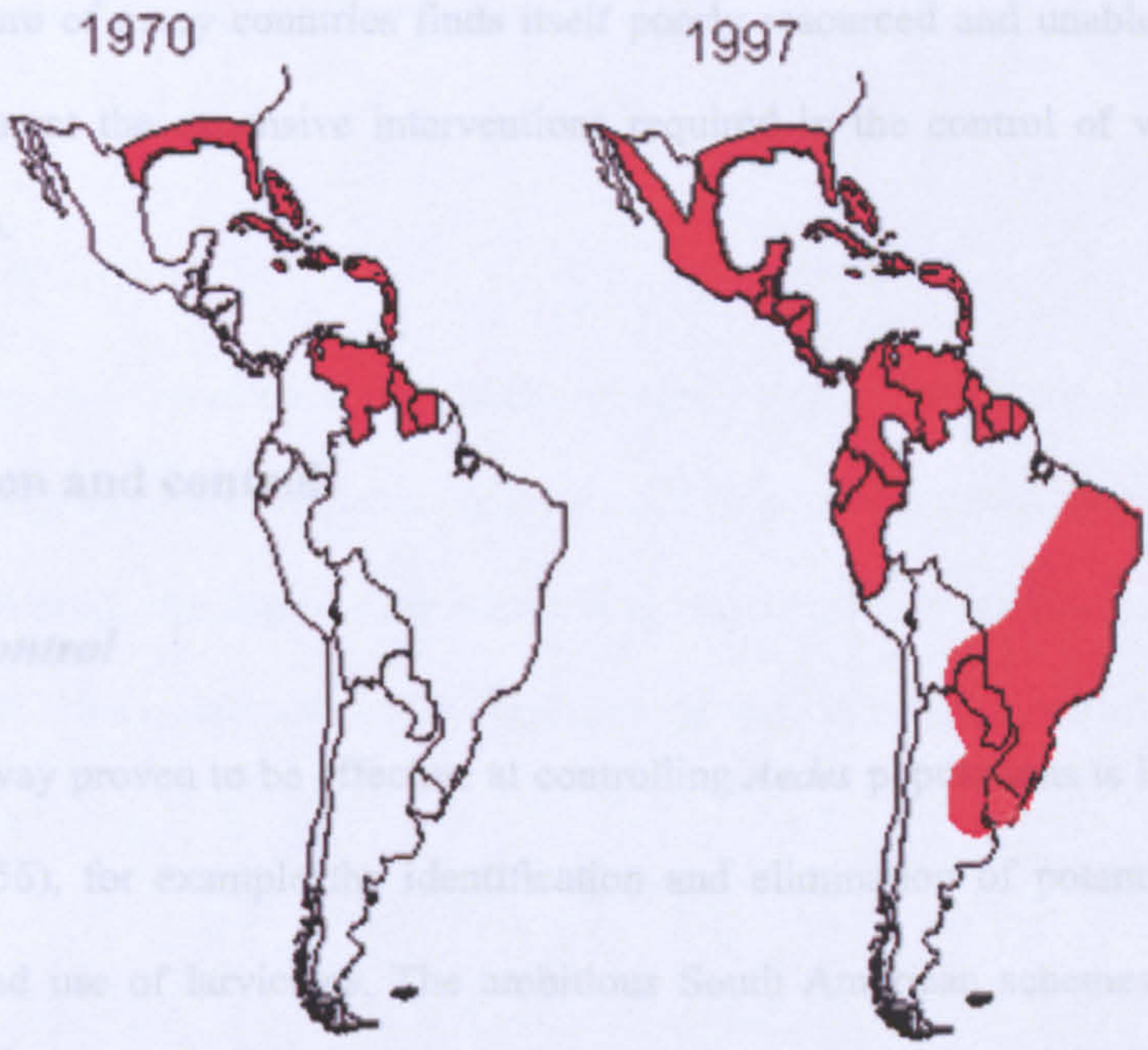


Figure 9. Distribution of Aedes aegypti in the Americas (source: Centres for Disease Control and Prevention, Atlanta, USA)

Epidemic DF has been reported throughout East and West Africa. Reporting is unreliable but to date whilst there have been sporadic cases of DHF there have been no epidemics. It is not certain whether this relates to the focal nature of the viral serotypes present in geographical areas or to genetic factors (46, 51).

In conclusion several important factors can be identified in the rise of DF/DHF as a public health problem. The reinfestation of the American tropics by *Aedes* brought the viruses into contact with a large, non-immune, urban population. The resulting epidemics facilitated the movement of viruses between regions and countries. Major global demographic changes following World War 2 (urbanisations, movement of people to the cities, towns encroaching on forests) have brought mosquitoes and man into closer and more intense contact. The city provides numerous larval friendly environments. And at a time when they are perhaps most needed the public health infrastructure of many countries finds itself poorly resourced and unable to manage and implement the expensive interventions required in the control of vector borne diseases(3).

Prevention and control

Vector control

The only way proven to be effective at controlling *Aedes* populations is larval source reduction(56), for example the identification and elimination of potential breeding habitats and use of larvicides. The ambitious South American schemes of the 20th century were almost paramilitary in scale and ultimately proved unsustainable. Once

mosquito numbers were controlled resources were diverted to other needs and the *A. aegypti* population quickly returned to levels high enough to permit epidemic transmission(56). The emphasis of many government departments of pubic health is now on the emergency response to epidemics rather than prevention of such epidemics. Despite this few new mosquito control methods have been developed in the past 30 years and most countries have had ineffective mosquito control programmes for the last 30 years or more(58). The population density and geographic spread of *Aedes* continues to increase, particularly in urban areas of the tropics (due to the prevalence of good mosquito larval habitats in the domestic environment – e.g. water containers and disused tyres) and the recent re-emergence of dengue in Singapore suggests that vector control is not an effective long-term strategy. After 15 years of low-incidence dengue has been re-emerging in Singapore despite its highly effective control programmes. This has been attributed to lowered herd immunity, virus transmission outside the home, an increase in the age of infection, and virus importation from neighbouring regions(59).

Surveillance

Active disease surveillance by both national public health departments and international bodies is an important component of a dengue prevention programme. It provides an early warning of outbreaks and provides information that may allow the prediction of future outbreaks and the initiation of early effective mosquito control.

Vaccines

Given the difficulties in both implementing and sustaining long-term vector control the development of a successful dengue vaccine remains the best hope of effective control(60). Although several are in development, none is yet available. Before discussing vaccines in detail it is useful to turn first to what is known about the pathology of dengue haemorrhagic fever.

The pathology of dengue haemorrhagic fever

The pathophysiology of DHF is poorly understood. Although mice and primates may be infected with dengue there is no model for DHF – the phenomenon of shock is not seen. In this section we will consider what is known about the virus, the pathological changes seen in infection and the nature of the immune response in order to gain an overview of what is understood about the mechanisms that lie behind the wide range of clinical severity seen in dengue infection.

The virus(3)

There are four serologically related but distinct dengue viruses designated DEN-1 to DEN-4. They are single stranded positive RNA viruses belonging to the family *Flaviviridae.* All the flaviviruses are spherical particles, around 40-50nm in diameter with a lipid envelope enclosing a nucleocapsid core. The envelope is fringed with surface projections – the envelope and membrane structural proteins. The genome is within the nucelocapsid, around 11kb in length and encodes an uninterrupted open reading frame flanked by 3' and 5' non-coding regions. The open reading frames vary slightly in length and encode a polyprotein precursor of 3396, 3391, 3390 and 3386

amino acids in length in DEN-1, -2, -3 and -4 respectively. The order of the proteins within this precursor is the same for all flaviviruses (Figure 10). The 5' quarter of the genome encodes the capsid (C), premembrane (prM, precursor of the mature membrane protein) and envelope (E) structural proteins. The non-structural protein genes take up the remainder of the genome (NS1 to 5). The polyprotein is co- or post-translationally processed into the mature viral proteins, probably by both host and viral proteases.

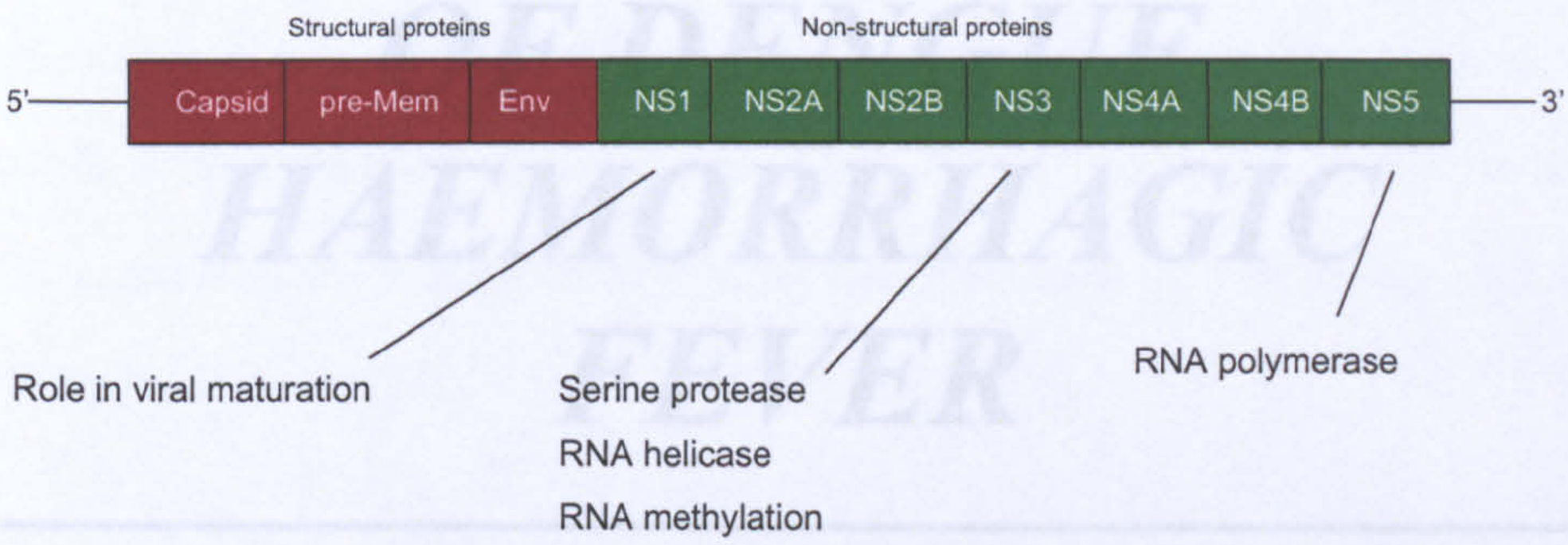


Figure 10. The polyprotein encoded by the dengue genome

- *Capsid* – the capsid is a small positively charged protein (112-127 amino acids). Its high proportion of basic amino acids is believed to help neutralise the negatively charged viral RNA with which it is associated(61). Although the amino acid homology between flaviviruses is low all conserve the hydrophobic nature of the peptide.
- *Membrane* – the immature form, pre-membrane (prM), is a glycoprotein contained in immature intracellular virions. It is 18.1-19.1 kDa and is cleaved into a 7-9 kDa mature form (membrane, M). This cleavage results in the rearrangement of the oligomeric structures on the surface of the virion and it promotes the infectivity of the mature viral particle. The immature prM is

thought to be important in protecting the E protein from irreversible conformational change in the acidic compartments of the secretory pathway before viral release.

- *Envelope* – this protein of 494-501 amino acids is the major component of the virion surface and is usually glycosylated. It is believed to have a role in the dissociation of the nucelocapsid after acidic-mediated fusion of the viral and endosomal membranes following virus uptake by receptor mediated endocytosis. As well as receptor binding its biological activities include haemagglutination of erythrocytes, induction of the major neutralising antibody in the protective immune response and virus assembly.
- *NS1* – a glycoprotein 353-354 amino acids in length. It exists in different forms in different locations. The functional form is believed to be a dimer (found in the intra and extracellular fluids of virus infected cell cultures) but its role in replication is unknown. It is thought to play a role in viral maturation. It is expressed on the surface of infected cells and has been identified as the soluble complement fixing antigen. The secreted form can elicit antibodies with complement fixing activity.
- *NS3* – highly conserved among flaviviruses this protein is 618-623 amino acids long. It is thought to act as a serine protease and helicase. A region near the N-terminus is required, in combination with NS2B for proteolytic processing at the dibasic site of many viral proteins. The rest of the protein shares homology with the RNA helicase superfamily.
- *NS5* – another protein highly conserved between flaviviruses and 900-905 amino acids in length. It is has an RNA-dependent RNA polymerase activity and may

also be involved in RNA viral capping. Both NS5 and NS3 are required for viral RNA replication.

- *The small non-structural proteins* – NS2A, 2B, 4A and 4B are poorly conserved in sequence between flaviviruses but retain their hydrophobicity profiles – they are likely to be membrane associated proteins. The functions have not been elucidated in detail but they appear to have roles in membrane localisation of the other proteins, and are involved in their processing and function.

Viral infection

Most animal viruses enter their host cells by receptor-mediated endocytosis(62) in clathrin-coated vesicles(63). Certain receptors utilised by the virus may be dengue-specific – certainly some are thought to interact fairly specifically with the E protein, the viral component mediating binding(64) – but the first-line dengue virus receptors are thought to be more generic: GAG (glycosaminoglycan) receptors and DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin). These are capable of binding a wide range of different viruses. Thought also to be particularly important in secondary infection is the binding of virus-IgG complexes to Fc receptors. What is not clear is why this interaction, which should normally place viruses in a lytic pathway, permits enhanced infection. In the course of natural infection dengue virus is probably inoculated by the mosquito into the subcutaneous or dermal space. Initial replication probably occurs at the site of infection in cells of the reticuloendothelial system and/or fibroblasts. Human monocytes and macrophages appear to be the primary cell infected(65). Immature DCs in the skin (Langerhan's cells) are also infected via DC- or L-SIGN(66). Virus-neutralising antibodies bind to the E protein.

Non-neutralising antibodies, or antibodies diluted beyond the neutralising end-point may enhance infection.

Once inside a cell uncoating of the nucleocapsid occurs by acid-dependent fusion of viral and endosomal membranes. Uncoating complete, replication is thought to proceed with immediate translation of the viral genome. The translated polyprotein is processed and the viral proteins produced. E and prM are inserted into the rough ER membranes during protein synthesis and transported to the Golgi apparatus for further processing and the addition of the oligosaccharide side chains. The nucleocapsid probably acquires its envelope through a process of budding in which viral particles assemble in the rough ER, are transported to the Golgi, and then carried within secretory vesicles to the cell surface.

Virus can be detected in regional lymph nodes 24 hours after inoculation. In humans viraemia occurs at about the time of symptom onset. By the time of DHF/DSS viraemia may no longer be detectable by culture due to the presence of antibody. It may be possible to isolate dengue virus from PBMC even after it is undetectable in the serum(67). Dengue antigen has been detected by immunofluorescence and immunoperoxidase methods in: reticuloendothelial cells of the spleen, thymic cortex, Kupffer cells and flat sinusoidal lining cells of the liver; alveolar macrophages; mononuclear phagocytes of the skin; up to 1% of circulating monocytes; B lymphocytes; the bone marrow element; platelets (perhaps contributing to complement mediated destruction and thrombocytopenia). Viral genome has been detected most consistently in liver cells.

Histopathology

With a few exceptions patients present with thrombocytopenia, coagulation disorders and a vasculopathy – obtaining tissue samples from living patients with invasive techniques is rarely feasible. Most information regarding gross pathology has come from post-mortem studies. The most prominent feature is plasma leakage and levels of total protein and globulin in plasma decrease in patients with DHF. Serous effusions with high protein content (greater than 4g/dl) are often found in the pleural, peritoneal and (occasionally) pericardial spaces. Chest X-rays demonstrate pleural effusions in around 70% of non-shock cases and almost all patients with DSS(12). Effusions are not generally haemorrhagic and although there is swelling of capillary endothelial cells the relatively low key tissue damage observed in pathologic studies is not in keeping with the severity of the illness(68). Haemorrhage appears as petechial rash (often particularly obvious on the legs) or purpura, especially around needle puncture sites. Bleeds may occur in the mucosa of the nose, gums and gastrointestinal tract as well as within the liver capsule. Frank bleeding into serous cavities is however unusual. Those patients experiencing prolonged severe shock (especially young adults) may develop the general pathological features associated with shock itself such as disseminated intravascular coagulation and more extensive haemorrhage.

Thrombocytopenia

Thrombocytopenia is a common feature of DF and always found in DHF/DSS. Its pathogenesis is poorly understood. Dengue-virus induced bone marrow suppression with reduced platelet synthesis has been suggested as one cause(69). Others have noted that DEN-2 is able to bind human platelets in the presence of virus specific

antibodies and suggest that this may lead to immune mediated platelet clearance(70). IgM anti-platelet antibodies have been identified in the context of dengue infection(71) and titres are higher in patients with DHF/DSS than those with DF. Certain antibodies directed towards dengue virus proteins (e.g. NS1) have been found to show platelet cross-reactivity – it has been suggested that such autoantibodies may have a pathogenic role(72) but this would fail to explain the rapid rise in platelet counts following viral elimination. Platelet counts do not correlate well with clinical bleeding and thrombocytopenia is not the cornerstone of haemorrhage pathogenesis(73).

Coagulopathy

Coagulation parameters such as platelet counts, activated partial thromboplastin time, as well as fibrinolytic parameters are altered in dengue infection. Coagulation and fibrinolysis pathways are activated, and more severely in DHF/DSS than DF(74). A recent review has however highlighted the inadequacy of studies attempting to link the extent of coagulation and fibrinolytic activation to disease severity and outcome(75). Certainly in dengue cases without circulatory collapse haemorrhage does not correlate with platelet counts or the severity of pleural effusions and although measures such as APTT are prolonged and fibrinogen levels depressed neither do these correlate with the presence or absence of significant haemorrhage. These observations lead the authors of one study to propose the principal mechanism of haemorrhage to be platelet activation(10). Adolescents and young adults with severe haemorrhage have evidence of intravascular clotting in the small vessels. Some degree of DIC has been observed in just over half of non-shock dengue patients. It is generally of only mild to moderate severity and severe DIC is seen in those patients

experiencing severe intractable shock with acidosis and fulminant hepatic failure(76). DIC may be averted by the early management of shock.

Vasculopathy

The characteristic feature of DHF/DSS and a better indicator of disease severity than haemorrhage is that of plasma leakage. This is caused by a diffuse increase in capillary permeability manifesting as haemoconcentration and fluid collections e.g. pleural effusions, ascites. It usually becomes evident around day 3-7 of illness – the time at which cases of DF resolve(12, 68). Plasma leakage occurs systemically, progresses quickly but resolves within 1-2 days in those managed appropriately. Perivascular oedema is obvious but there is no destruction of vascular endothelial cells – leakage appears to be due to altered vascular permeability. This functional alteration could be due to structural damage of the vessels or the release of cytokines or other inflammatory mediators during dengue infection.

- *Structural changes* - dengue virus is capable of infecting endothelial cells *in vitro* (77) and certain viral strains may have a cytopathic effect(78). Although apparent experimentally such cell damage has not been demonstrated in vivo to a very great extent – it may be that only very subtle damage is required to cause significant leak. Infected endothelial cells can activate complement and upregulate the expression of adhesion molecules (e.g. ICAM-1) and it is possible that a combination of direct viral cytopathic activity and immune-mediated damage by leucocyte recruitment and anti-dengue virus antibodies contribute to structural injury of infected endothelial cells(79). NS1, as noted above, is an inducer of cross-reactive antiplatelet antibodies, also stimulates the production of antibodies that lead to endothelial cell activation, damage and apoptosis(80, 81).

Some researchers take the extreme view that the immunopathology of dengue is essentially autoimmunity(73).

- *Cytokines* - capillaries and venules of affected organs may show perivascular haemorrhage with a lymphocytic/mononuclear infiltration yet destruction of vascular endothelial cells is not often apparent and dengue virus antigens are not consistently detected in endothelial cells. The rapidity of shock onset and its systemic nature belie the speed of recovery and the low mortality of well managed cases. A process that mechanically damaged endothelial cells, such as that seen in other more classical viral haemorrhagic fevers, would not be expected to behave in this manner. Plasma leakage is likely to be due to altered permeability rather than endothelial cell destruction. Human microvascular cells cultured *in vitro* with sera from patients with acute dengue infection increase their expression of ICAM-1 and undergo apoptosis – an effect more pronounced with serum samples taken during the acute febrile phase than those from the convalescent phase of the disease. This effect appears to be due largely to TNF-α as the endothelial activating effect of acute dengue sera was inhibited up to 80% by pre-treatment with monoclonal antibodies against TNF-α(82). Mononuclear cells are highly activated during infection and as such it is not surprising that many cytokines are present in serum at elevated levels during dengue infection. Studies have identified IL-2, IFN-γ, TNF-α, and GM-CSF as being raised in children with dengue infection, and these markers were higher in DHF/DSS patients than DF patients(83-87). Other studies have also noted raised levels of type 2 cytokines such as IL-10, IL-13 and IL-18(88, 89), with IL-10 levels in particular found to correlate with disease severity(90). *In vitro* infection of human PBMC with dengue virus results in a type 1 cytokine response during the

first 3 days that is replaced by a type 2 response from around day 4(91). This change from type 1 to type 2 cytokine production has been observed clinically, taking place at around the time of defervescence(92). It may be that the inflammatory host response to dengue infection is followed by the generation of inhibitory cytokines to counteract the inflammation with the balance between the two influencing the outcome(79). TNF-α is the factor largely responsible for endotoxic shock. It is produced by monocytes/macrophages and T cells, increases vascular permeability and is directly toxic to vascular endothelial cells. Administration of TNF-α induces microvascular protein leakage and shock(93). IL-2 is produced mainly by T cells and its administration can cause capillary leak, thrombocytopenia and complement activation (94). IFN-γ is produced by T cells and increases endothelial cell permeability *in vitro*. These various cytokines often have synergistic effects giving an increase in permeability in combination greater than each cytokine alone. Some cytokines which exert no permeability effect on their own increase that of others when combined(95).

Histopathology of specific organs

- *Lymph* – lymphoid tissue has evidence of plasma cell proliferation with active germinal centres and an increase in the number of B lymphoblasts.
- *Central nervous system* – there may be perivascular oedema of the brain and spinal cord but signs of encephalitis are extremely rare, despite the frequency of "encephalopathic" symptoms(3).
- *Gastrointestinal tract* – may be oedematous with haemorrhage into the mucosa, submucosa and serosa. Those patients who experience prolonged shock may have marked congestion and haemorrhage.

- *Bone marrow* – during the febrile period there is bone marrow depression affecting most blood elements but samples taken at the time of shock appear normo- or hyper-cellular. Megakaryocytes proliferate and may be found in the capillaries of viscera.

- *Skin* – biopsy of skin rashes show that the microvasculature located in the dermal papillae is the main site of injury with swelling of the endothelial cells and perivascular tissues. Some red cells may be seen outside the vessel wall. Immunoglobulin (mostly IgM), complement and fibrinogen are found on the vessel walls. Dengue antigen can be demonstrated in the cells surrounding the microvasculature(96). There is no evidence of necrosis of the vessel wall or vasculitis. Electron microscopy of the skin microvasculature shows non-specific changes indicative of increased transport activities by endothelial cells. Dengue virus has not been detected in any skin cells by electron microscopy(97).

- *Liver* – dengue is hepatotropic and viral antigen has been detected in hepatocytes. The virus can infect liver cells directly and cause hepatitis. Serum transaminase levels are raised in dengue patients and the AST (aspartate aminotransferase) level correlates with haemorrhage(98, 99). The reduced levels of coagulation factors seen in dengue may reflect impaired synthesis due to liver injury. The liver can be enlarged with histological changes indicative of shock – parenchymal bleeds are uncommon(68). The liver undergoes changes similar to those seen in experimental yellow fever infections and also recognised in other viral haemorrhagic fevers such as Lassa, Ebola and Marburg. There is focal necrosis in the paracentrolobular or midzonal regions with swelling and hyaline necrosis of Kupffer cells. Mononuclear leucocytes may be seen in the sinusoids

and portal areas. Liver changes may be extensive and associated with clinical jaundice.

The immune response to dengue infection

The innate immune system provides the first line of defence against viral infection, mounting early responses and initiating and directing the action of other immune system components. The adaptive immune response may take a few days to become truly effective and the innate system is vital for controlling infectious processes during this time(100). The adaptive immune arm is more versatile and capable of generating immunologic memory such that subsequent encounters with the same infectious agent are mounted more rapidly and effectively. Although adaptive responses fall into humoral (antibody-mediated, B-lymphocyte effectors) and cellular components (cell mediated, T-lymphocyte effectors) they interact with and influence each other.

The innate immune response to dengue infection

It is the components of the innate immune system that are involved in the earliest stages of dengue virus infection. Interstitial dendritic cells (DCs) are thought to constitute the first line of innate defence at the anatomical sites where dengue virus replicates after an infected mosquito bite(101). Dendritic cells in the peripheral tissues capture antigens, process them into immunogenic peptides and emigrate to the draining lymph nodes in order to present them to T cells in the context of HLA class I or II. Immature monocyte-derived dendritic cells generated *in vitro* have been shown to be ten times more permissive to dengue virus infection than even macrophages or monocytes themselves(102). Histological analysis of skin biopsies from human volunteers inoculated with live-attenuated dengue vaccines has shown that viral

replication is supported in DCs. Productive infection occurs in immature myeloid DCs and activated DCs secrete TNF-α and IFN-α in response to DV infection(101, 103, 104). Infection is inhibited by blockade of DC-SIGN (dendritic cell ICAM-grabbing non-integrin, a receptor on the DC surface)(105). Early activation of NK cells may be important in the clearance of primary dengue infection(106) – they exhibit cytotoxic activity against dengue infected cells(107). Type 1 interferon is also important in inducing an antiviral state in as yet uninfected cells, and limiting viral replication in the early stages of infection. Along with the IFN system, cytokines and chemokines, myeloid DCs and NK cells might play a key role in orchestrating the initiation of the adaptive immune response, with the subsequent activation of effector B and T cells.

The humoral immune response to dengue infection

Antibodies and B cells

Antibodies consist of two identical heavy chain molecules bound to two identical light chain molecules by a disulfide bond. The N terminals of each chain possess a variable domain that is able to bind antigen through three hypervariable complementarity determining regions. The C terminal domains form the constant regions – these define the class of the antibody: IgG, IgA, IgM, IgD or IgE. The classes have different functions and each antibody may be found freely circulating or anchored to the cell membrane (i.e. the B cell receptor, BCR) by the addition of hydrophobic transmembrane sequences. The antigen binding region (the Fab portion) is bivalent with two antigen-binding arms of identical specificity. The Fc portion contains most of the constant region of the heavy chains. After deletion of B cell producing self-reactive antibodies it is estimated that the B lymphocyte population is capable of

producing 10^{15} different antibody specificities(108). They produce these from a limited number of genes by combining different genes (combinatorial diversity) in different ways (junctional diversity) and mutating this combined DNA in mature B cells (somatic hypermutation). There are no more than a few thousand lymphocytes specific for each antigen. Each B cell can produce only one of the enormous number of potential antibodies, thus all the BCRs on a given lymphocyte have the same specificity. If the BCR meets its antigen the cell is selected and begins a process of division, rapidly expanding in number. Most responses will involve many different B cell clones as a virus or protein will bear several different epitopes each with the capacity to bind a different clone (Figure 11). In addition each epitope is likely to be recognised by different B cell clones in slightly different ways. The genes encoding BCRs undergo somatic hypermutation during B cell proliferation within secondary lymphoid organs. This process fine-tunes BCR recognition and alters the binding affinity of the antibody for the antigen. This results in populations of B cells with high affinity and specificity for the immunising antigen which also constitute the memory of the exposure. These memory cells enable a faster and stronger secondary immune response on further encounters with the same (or as we shall see a related) antigen.

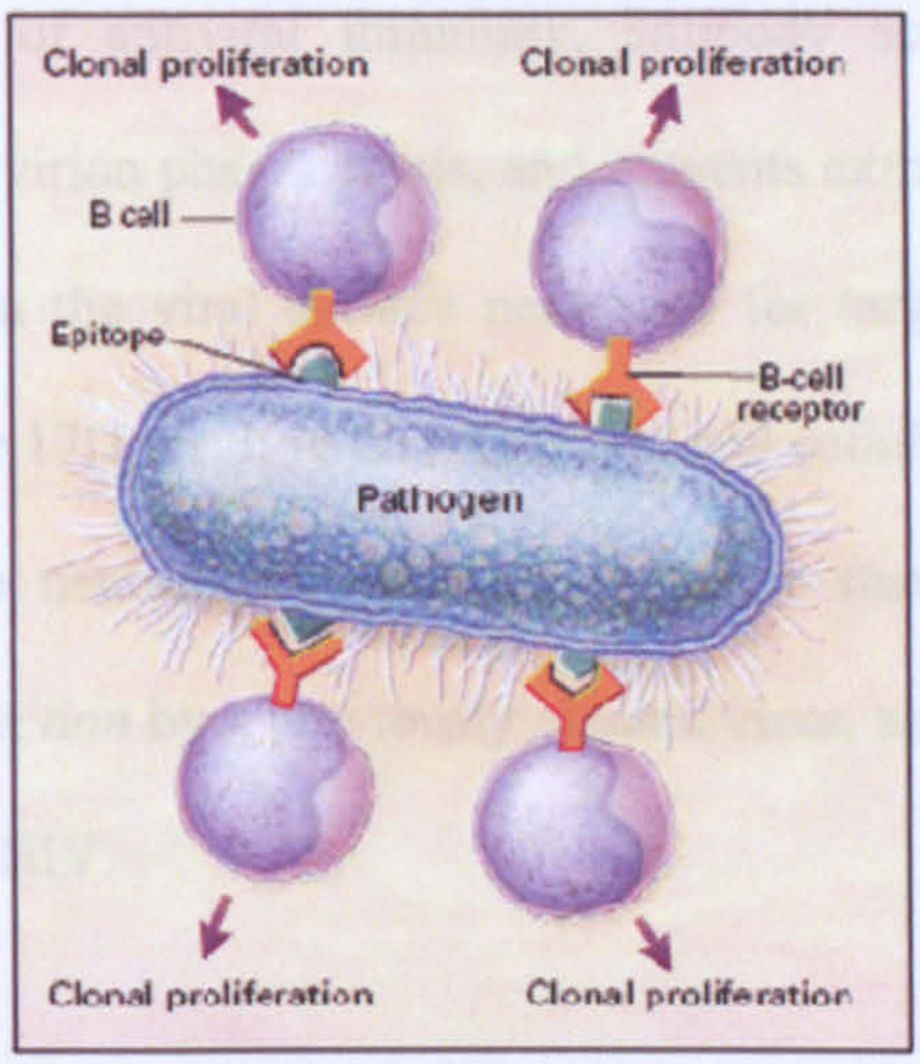


Figure 11. B cell responses to antigenic stimulation. Using the antibody molecule as its receptor, the B cell recognizes epitopes on the surface of the antigen. If it is stimulated by this contact, the B cell proliferates, and the resulting clones can secrete antibody whose specificity is the same as that of the cell-surface receptor that bound the epitope. Responses usually involve several different clones of lymphocytes and each epitope may be recognised by several different lymphocyte clones with different B-cell receptors, each recognising the epitope in a slightly different way.

Naïve B cells coexpress IgM and IgD on their cell surface. On encountering their antigen B cells proliferate within germinal centres of secondary lymphoid tissue and undergo class-switching, producing IgG, IgA or IgE. Somatic hypermutation also takes place within the receptor genes. The B cells differentiate into memory cells and antibody-secreting plasma cells. By the time they have become memory cells they have usually switched to the use of IgG, IgA or IgE as their receptor.

In general antibodies bind to specific epitopes through the Fab portion but do not have a direct effector function. Instead they target pathogens for destruction by other immune system players. For example, antigen-antibody complexes activate the complement cascade through the classical pathway, and binding of the macrophage FcγR receptors by the Fc portion of IgG opsonising antibodies promotes pathogen phagocytosis(109). Thus although cellular immune responses are often thought of as

being the cornerstone of antiviral immunity, antibody activates direct lysis by complement, facilitates virion phagocytosis, and prevents extracellular viral spread by blocking receptors upon the viral surface necessary for target cell entry. Vaccines such as the yellow fever 17D result in both humoral and cellular immunity(110) and it is likely that it is the neutralising antibody response that is most important in protection against *reinfection* by a previously cleared virus, as opposed to control of a persistent virus such as HIV.

The humoral response to dengue

Dengue virus's E glycoprotein, the major virion surface protein, is the most important antigen with regards to virus biology and humoral immunity. It is largely responsible for virus attachment to susceptible cells and mediates virus-specific membrane fusion – allowing newly infecting virus to escape the endocytic vesicle and initiate intracellular replication. It elicits virus neutralising antibody, haemagglutination-inhibiting antibody, anti-fusion antibody and virus-enhancing antibody. It also plays a role in the cell-mediated immune response. Different monoclonal antibodies have been generated that recognise the E protein in flavivirus/DEN serotype specific and cross-reactive manners. CD4+ and CD8+ T cells have also been shown to recognise parts of the E protein in either serotype-specific or cross-reactive manners. Most T cells however tend to recognise the non-structural proteins. Little is known about the B or T cell response to the M or C protein. NS1 is a major surface non-structural protein. It elicits an antibody response and contains T cell epitopes.

Passive transfer experiments have demonstrated that dengue virus antiserum protects mice from lethal dengue infection. Experiments with monoclonal antibodies in mice

have shown that they act through a combination of virus neutralisation, complement lysis, and antibody-dependent cellular cytotoxicity(14, 111). Antibodies to non-structural proteins lack neutralising activity, yet comprise a major part of the antibody response to dengue virus. Monoclonal antibodies to non-structural proteins have been shown to exert a protective effect(111, 112). Antibody mediated activation of the classic complement pathway occurs during severe dengue and can occur less commonly in primary infection (113).

Antibody dependent enhancement

The phenomenon of antibody-dependent enhancement (ADE)(114, 115) is widely accepted as a good explanation of the link between severe dengue disease and evidence of previous exposure. Some viruses are able to use pre-existing antibodies which *should* act to neutralise their ability to infect through natural receptors but instead facilitate infection through binding to the Fc or complement receptors on viral target cells. Such viruses usually replicate in macrophages or monocytes and then go on to infect other tissues. The increased viral productivity may lead to exacerbation of disease(116). The phenomenon was first noted in the 1960s when it was observed that high dilutions of homologous antibody – neutralising at low dilutions - increased the yield of a variety of flaviviruses generated in chick embryo cell culture(117). It was not until the late 1970s that the concept was linked with severe dengue disease(8). ADE has since been observed in HIV-1, RSV, Hantavirus, West Nile virus and Ebola among others. ADE is thought to be a disease-enhancing factor for several human diseases including dengue and RSV. Strong and legitimate concerns surround the development of vaccines for such viruses. The introduction of an experimental RSV vaccine in the 1960s was associated with a dramatic increase in cases of severe

pneumonia among those subsequently infected with RSV (from 4% of unvaccinated patients to 69% of vaccinated). The exact mechanism of the effect is not clear but it appears the vaccine induced only low levels of neutralising antibody(118).

Features predisposing a virus to ADE include:

- The capacity to replicate within macrophages.
- The induction of a large amount of antibody that is poorly neutralising for even homologous virus.
- The tendency to a prolonged viraemic phase of disease.
- Membership of a group of viruses with some antigenic diversity rendering them partially resistant to neutralisation by antibody raised against heterologous viruses(116).

What is puzzling is that the interaction of virus-antibody complexes with FcR on monocytes/macrophages or granulocytes usually results in the antiviral responses of phagocytosis, cytokine release and antibody-dependent cell-mediated cytotoxicity. It is not clear how infection could be enhanced by this interaction. It is presumed that viruses are able to modulate antiviral responses and/or tend to infect immunologically immature subpopulations of these cells(119). Binding to the target cell in and of itself is not necessarily sufficient for infection. Other viral and cell proteins are likely to be involved in the internalisation process for some viruses. For such viruses the presence of antibodies that prevent binding would not neutralise infection as internalisation may occur through pathways other than the endocytic route. Animal work has shown that only IgG has enhancing activity in experimental systems – IgM does not enhance infection(120). Of the receptors known to play a role in ADE, the FcR is the most

important. Enhancement of West Nile virus infection of a macrophage cell line can be blocked by pre-treating cells with an anti-FcR monoclonal antibody(121). The proteins associated with ADE tend to be those on the surface – the envelope proteins.

Viruses in the same family may share common antigenic determinants, thus ADE of virus infections can be mediated by antibodies raised not only against heterologous strains but also against different serotypes or closely related viruses in the same genus or family. Enhancing antibodies may not be highly specific for a specific virus. For example, dengue infection can be enhanced by antisera raised against not only heterologous serotypes of dengue itself but antisera specific for other flaviviruses, suggesting that not only serotype-specific but also serotype- and flavivirus-cross-reactive epitopes are associated with ADE(116, 120, 122).

For some viruses homologous antibody appears to induce greater enhancement of infection than heterologous antibody (122). This might reflect the number of "enhancing" epitopes shared among viral species. Cross-reactive enhancing antibodies could be important epidemiologically in those areas where such viruses are endemic – they could promote the persistence of multiple serotypes in the population. The difference in "enhancing" epitopic profile between antigenically distinct strains of virus may influence the magnitude of ADE that results from the presence of antibodies directed against heterologous viruses. Strains may vary in their susceptibility to ADE and/or ability to induce ADE of infection(123).

ADE and dengue

It was Halstead's key epidemiological studies in the 1970s that demonstrated that the vast majority of cases of DHF were occurring in those experiencing secondary infection(7). The epidemiological observations that ADE seeks to explain are described above. Here we will examine some of the experimental data that supports the role of ADE in dengue pathogenesis.

Enhancement was originally demonstrated using dengue virus antisera mixed with virus at sub-neutralising concentrations(120). *In vivo* studies showed that the intravenous administration of dengue-immune human sera to rhesus monkeys shortly before infection with dengue virus significantly increases the viral load compared to monkeys given non-immune human sera(9). Monoclonal antibody studies have shown that enhancing antibodies are directed at the E or pre-M protein. These are generally not serotype specific but reactive across the serotypes and even across the flaviviruses(124). Studies with monoclonal antibodies have demonstrated that only certain non-neutralising antibodies are able to consistently enhance infection. Neutralising antibodies do not always mediate enhancement *in vitro* when diluted beyond their neutralising endpoint(125). Whether they do or do not appears to be dependent upon the viral strain(122). High levels of neutralising antibody protect patients from DHF/DSS whereas enhancement of infection can occur in the presence of low-level neutralising antibody(126). This becomes a concern in the development of a vaccine for dengue – as antibody titres wane post-vaccination it might be that the fraction of the total anti-dengue antibody pool which is neutralising drops below a protective level. This might permit enhancement of infection by the remaining antibody pool that recognises non-neutralising epitopes. Sera from dengue patients

can certainly enhance infection. A study examining the enhancement activity of pre-illness sera from Thai children with secondary dengue found that samples taken from those who later developed severe disease more effectively enhanced dengue 2 virus growth in human monocytes *in vitro* than that from those with mild disease(126). Another study found no correlation between the presence or absence of enhancing activity and viral load or disease severity(127). It may be that the enhancing effect is influenced by the viral serotype. Dengue virus isolates – both serotypes and specific strains within those types – certainly vary in their susceptibility to ADE mediated by antibody raised against heterologous serotypes or strains. *In vitro* assays have shown that DEN-2 infection is enhanced more effectively by DEN-1 antisera than by homotypic serum(120, 122). It is likely that different strains also vary in their ability to induce enhancing antibodies.

Antibodies elicited by other flaviviruses can enhance dengue virus infection *in vitro*(128). A study examining the efficacy of an experimental live attenuated DEN-2 vaccine noted that serum samples taken prior to vaccination from yellow fever-immune participants showed significantly greater *in vitro* enhancing activity than sera from non-immune subjects(129). Volunteers with yellow fever immunity developed viraemia a mean of 2.5 days earlier than the non-immune group(130).

Limitations of the ADE hypothesis

The evidence for ADE in dengue, both epidemiological and experimental, is good. The observation that perhaps more than any other it is difficult to explain by any mechanism other than ADE is that of DHF in infants between 6-9 months of age. For example, cellular mediated immunity is highly unlikely to be responsible for severe

disease in this age group. Those adopting a cautious approach to ADE would suggest that slightly different pathological processes are at work in different age groups. Certainly the microvascular permeability would appear to be higher in young children than in adults(41) and it might be that this group are more vulnerable to shock after the loss of maternal antibody (Figure 7). However it could be argued that changing vascular permeability does not adequately explain the abrupt decline in incidence of severe disease at around 9 months.

The ADE hypothesis relates disease severity to viral load: enhancing antibody facilitates infection of the target cells and the amount of virus produced is increased. The resulting pathology (be it a direct viral effect or an immunopathological mechanism) is correspondingly more severe than that seen in primary infection. Early studies failed to find an association between viral load and disease severity(131). However more recent work taking advantage of molecular techniques of viral detection has found that viral load is higher in patients with DHF than those with DF and – in contrast to those with DF – although falling rapidly in titre, virus remains detectable at defervescence (132, 133).

ADE may not be the whole story. The clinical syndrome tends to be at its most severe some time *after* the peak in viral load – virus is often undetectable at this time point(134) – suggesting that although ADE may indeed facilitate the high viral load it does not immediately provide a link to pathology. It is still rare for a patient experiencing a secondary infection to develop DHF (between 1.8 and 12% of such patients(28, 135)) and other factors must therefore play a role in pathogenesis. In addition not all cases of severe disease are seen solely in secondary infection. ADE is

neither sufficient, nor perhaps absolutely necessary for severe disease. It has been suggested that waning antibody levels may simply fail to effectively neutralise permitting the development of high viral titres – there is no need for antibody to have positively enhancing activity. This would not explain the relative absence of severe disease in primary infections however.

Whatever the mechanism by which a high viral load is achieved it does not in and of itself lead to DHF. The process by which enhanced infection might lead to the pathological features described above is not entirely explained by ADE. Other components are likely to be involved.

Cellular mediated immunity

Cellular immunity is mediated by antigen-specific T cells. Unlike humoral immunity this type of immunity cannot be transferred to naïve recipients with immune serum but requires the presence of specific immune cells(100).

The T cell receptor

It is the T cell receptor (TCR) that determines the antigen specificity of the cellular immune response. The TCR is a transmembrane heterodimer composed of either an α and a β chain or a γ and a δ chain, each containing a constant and a variable region. The TCR specificity is determined primarily by the sequence of three hypervariable complementarity determining regions (CDR) found within each chain (CDR 1-3)(108) and are discussed further below. α/β lymphocytes recognise antigen-derived epitopes presented by HLA molecules. The nature of γ/δ T cell antigen recognition is not so straightforward. Some appear to be able to recognise antigen in isolation, others

require presentation by "nonclassical" HLA molecules (e.g. CD1). They are capable of recognising a much broader range of antigen than α/β T cells including lipids and glycolipids, such as those found in mycobacterial cell walls. Despite their similarities to α/β T cells many researchers would consider them to be more properly part of the innate immune system(136) – they are not discussed further here.

The HLA System

There are two classes of HLA molecules, class I and II. They differ in structure and in function. Class I molecules are expressed on the surface of most somatic cells in the body. They consist of an α chain and a β2-microglobulin molecule. Class II molecules are expressed by more specialised groups of immune cells (e.g. dendritic cells, macrophages, B cells and activated T cells). They consist of an α and a β chain(137). These molecules are illustrated in Figure 12. In both cases the chains form a peptide-binding groove comprising a β-pleated sheet floor and two α-chain coiled walls. These walls pinch inwards in class I molecules (Figure 13) limiting the length of the peptide that can be accommodated within the groove to around 8-12 amino acids. The class II molecules has an open groove and is able to present much longer peptide fragments(137).

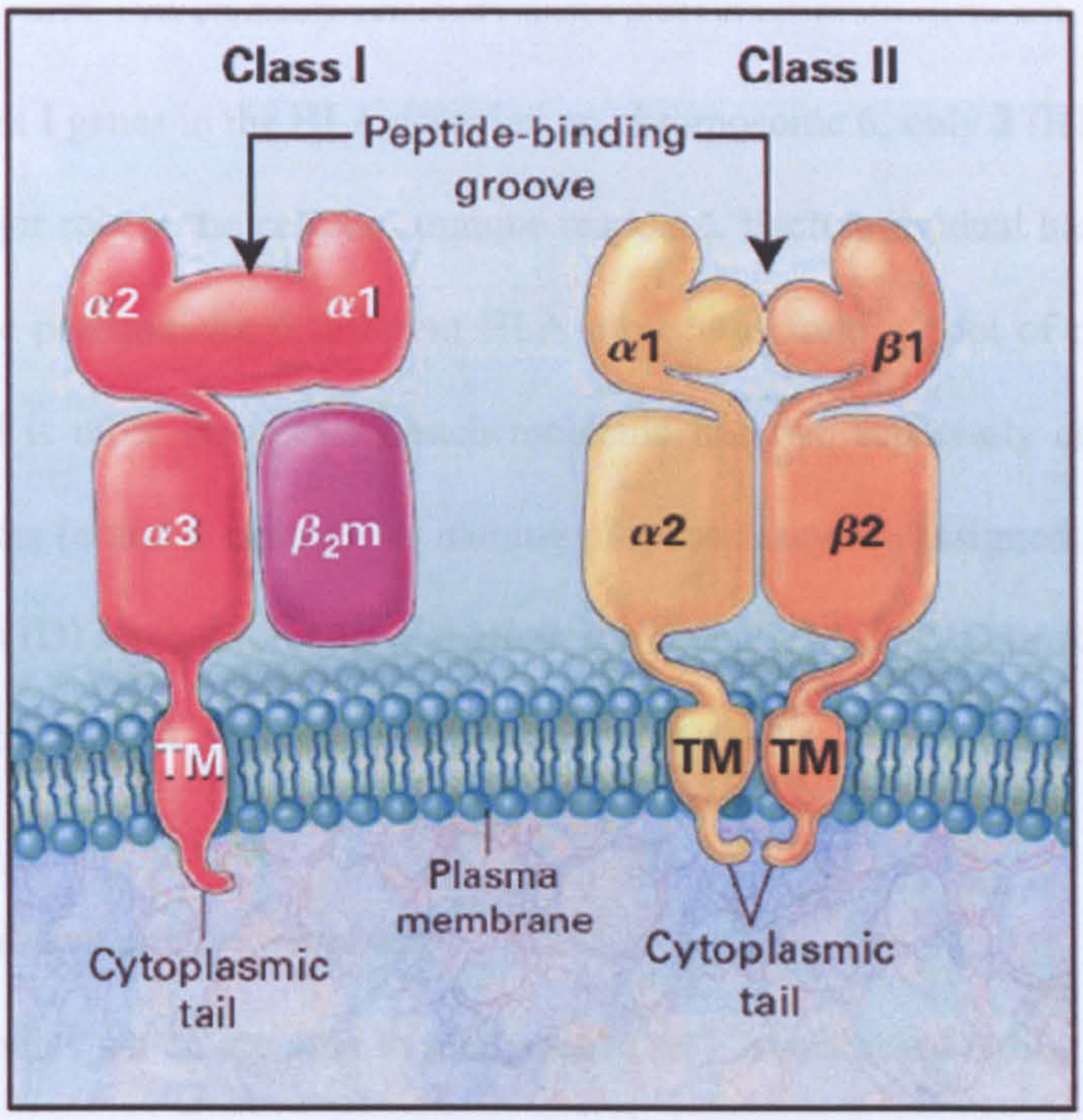
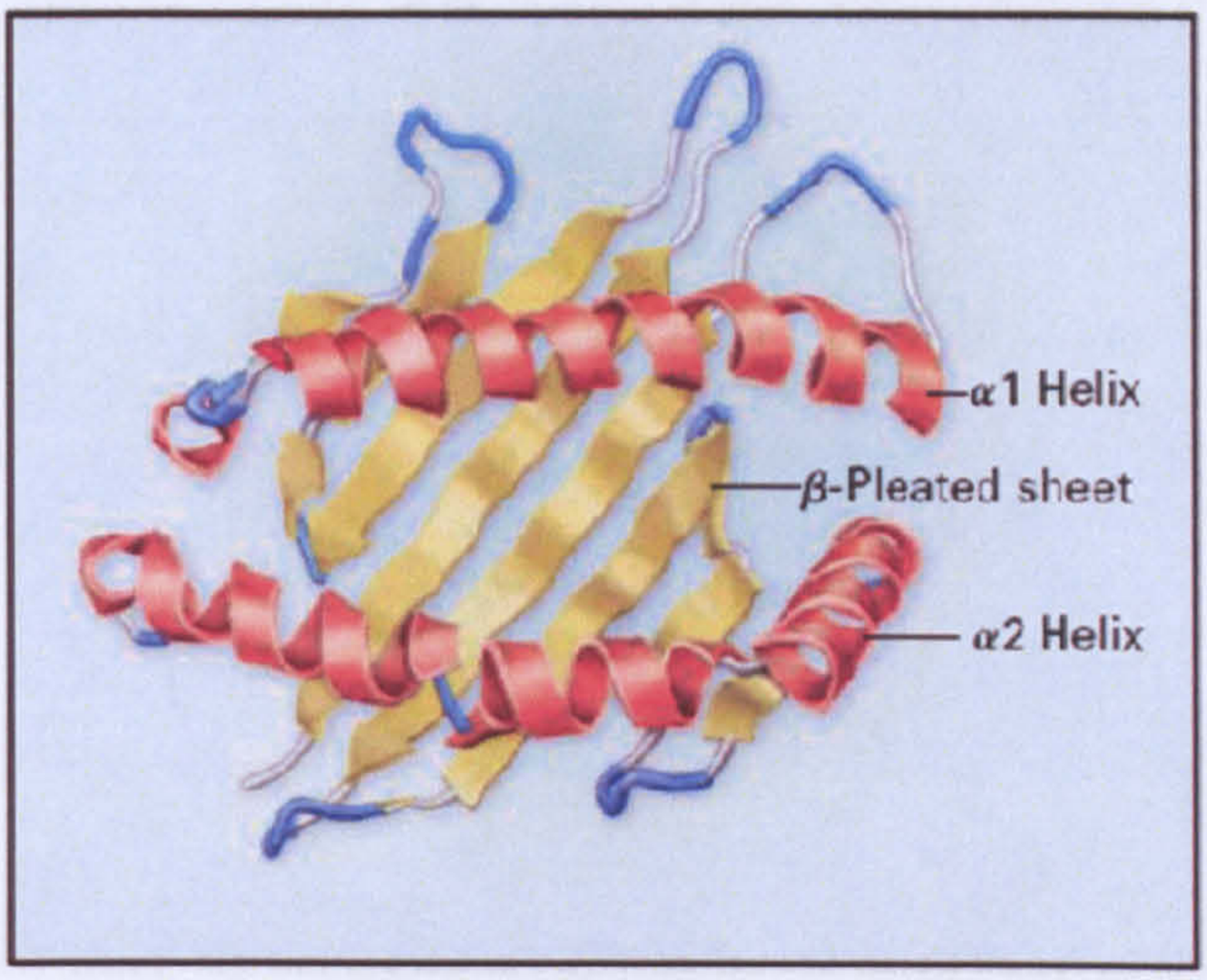


Figure 12. The structure of HLA Class I and II molecules. The α chain of the class I molecule has a peptide-binding domain (bound by α1 and α2 helices), an immunoglobulin-like domain (α3), the transmembrane region (TM), and the cytoplasmic tail. Each of the class II α and ß chains has four domains: the peptide-binding domain (α1 or ß1), the immunoglobulin-like domain (α2 or ß2), the transmembrane region, and the cytoplasmic tail. From Klein, J., and A. Sato. 2000. "The HLA system. First of two parts." N Engl J Med 343:702-709.

Figure 13. A schematic view of the HLA class I binding groove. The model is shown from the top. From Klein, J., and A. Sato. 2000. "The HLA system. First of two parts." N Engl J Med 343:702-709.



Of the 20 class I genes in the HLA complex on chromosome 6, only 3 (HLA-A, B and C) play a major role in the cellular immune response. Each individual has 2 copies of these with the potential for 6 different HLA molecules from a pool of many alleles. HLA class II is more complex as each molecule has two separately coded antigen binding regions (α and β chain). For naming purposes they are assigned a three letter code, the first (D) representing class II HLA, the second (M, O P, Q or R) the class II family, and the third (A or B) the α or β chain respectively.

Antigen processing and presentation

Unlike antibodies which are able to recognise "raw" unprocessed native antigen α/β T cells recognise antigen only if presented to them by the appropriate HLA molecule. Therefore the host must process antigens derived from a pathogen in such a way that they can interact with the TCR of pathogen-specific T cells. This processing generally follows one of two pathways (Figure 14).

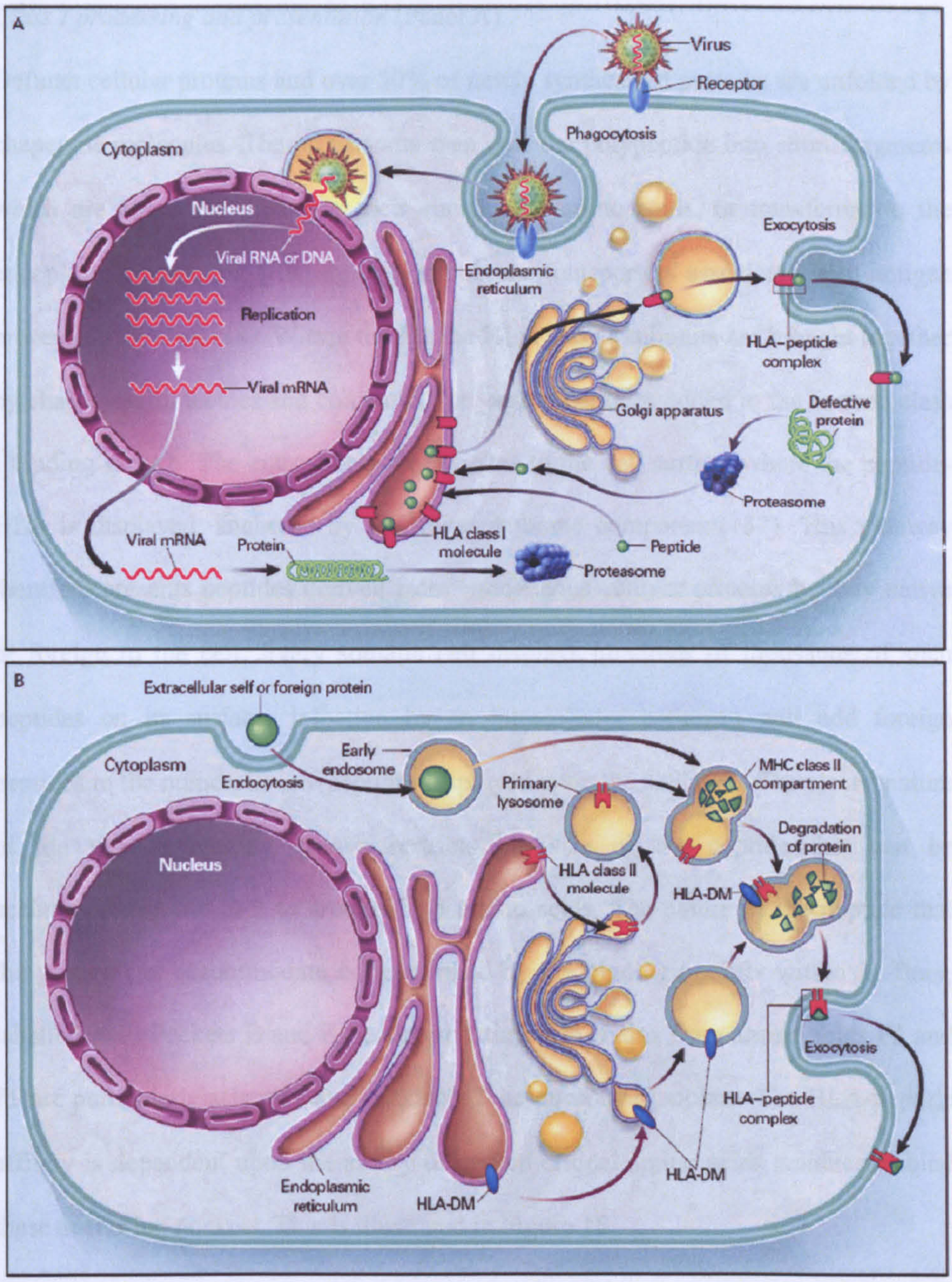


Figure 14. The pathways of generating peptides for loading onto class I (A) and class II (B) HLA molecules. From Klein, J., and A. Sato. 2000. "The HLA system. First of two parts." N Engl J Med 343:702-709.

Class I processing and presentation (Panel A)

Defunct cellular proteins and over 30% of newly synthesised proteins are unfolded by chaperone molecules. The proteasome then cuts the polypeptide into short fragments which are either recycled into their constituent amino acids, or transferred to the endoplasmic reticulum (ER) through membrane transporters associated with antigen processing (TAPs)(138). Within the ER the HLA class I subunits are brought together by chaperone molecules and compatible processed peptides added to the formed class I binding groove. The entire complex migrates to the cell surface where the peptide-HLA is displayed, anchored by the transmembrane component(137). This pathway therefore presents peptides derived from endogenous cellular proteins be they native or foreign to the cell. Every somatic cell displays hundreds of thousands of such peptides on its surface. Infection by an intracellular pathogen will add foreign peptides to the numerous self-peptides displayed upon the cell(139). The closed nature of the peptide binding groove restricts the size of the peptide that can be accommodated within it to around 7-15 amino-acids. The nature of the peptide that the groove can accommodate is determined by the binding pockets within its floor, labelled A-F. Pockets B and F, accommodating side chains from amino acids P2 and P9 are particularly selective and considered the *anchoring pockets*. The HLA-peptide affinity is dependent upon the ability of certain critical amino acids residues to bind these anchoring pockets. This is illustrated in Figure 15.

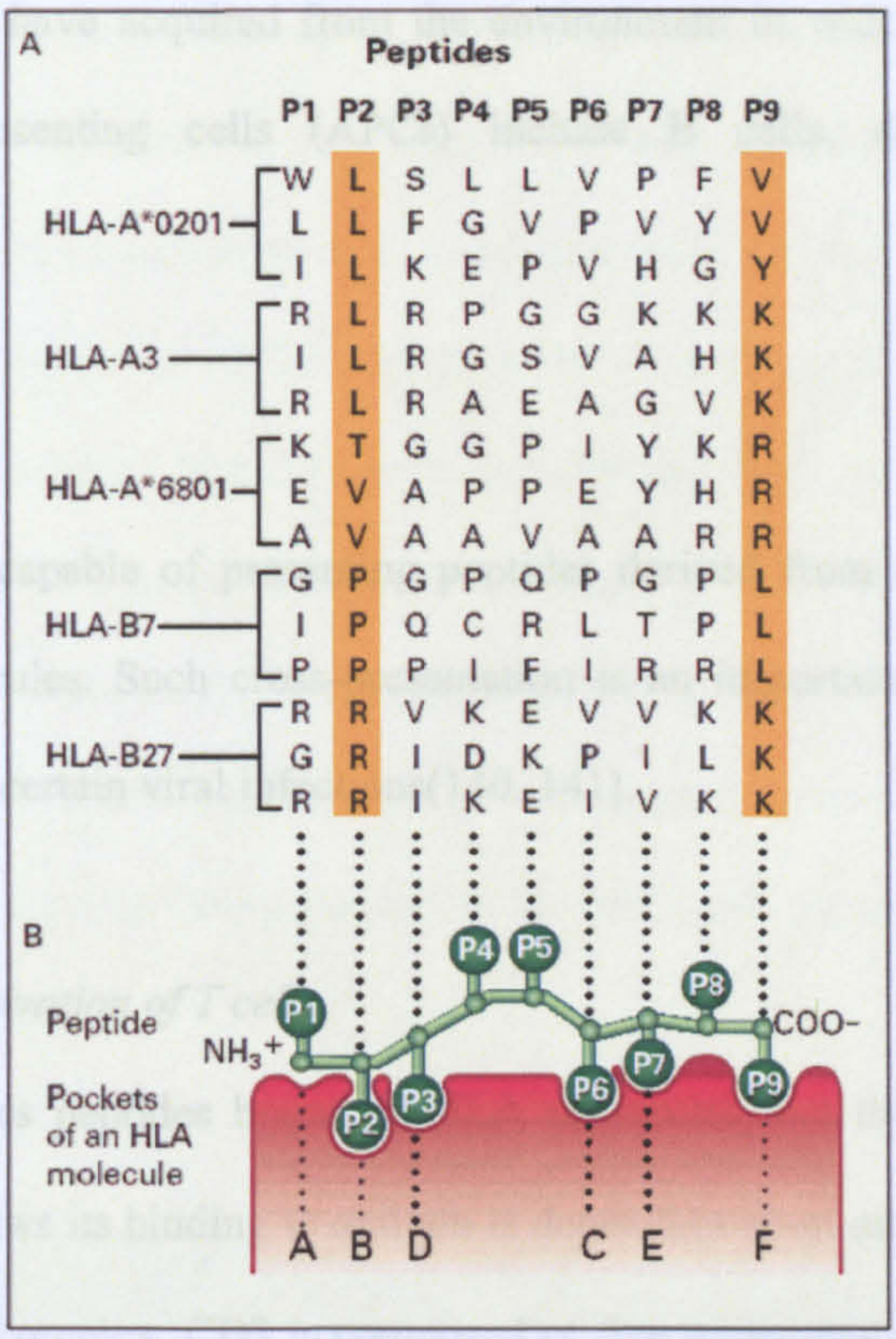


Figure 15. Examples of interactions between HLA molecules and peptides. A) The illustrated nonamer peptides have been found in complexes with the given HLA class I molecules. Anchor residues are highlighted. **B)** longitudinal section through the peptide's-binding groove demonstrates how the side chains of amino acids composing the bound nonamer are oriented either up or down into the pockets. From Klein, J., and A. Sato. 2000. "The HLA system. First of two parts." N Engl J Med 343:702-709.

Class II processing and presentation (Panel B)

Foreign proteins are taken up by the cell by endocytosis or phagocytosis. The class II HLA chains are brought together with the ER and become associated with the invariant chain, part of which serves to block the peptide binding groove and prevent premature peptide loading. These class II-invariant chain complexes are transported to the endosomes within which exogenous proteins are degraded by protease enzymes. The invariant chain is released from the binding groove and a suitable exogenous-protein derived peptide takes its place. The entire complex is then transported to the cell surface(138). This path allows certain specialised cells to present peptides derived

from proteins they have acquired from the environment in which they are located. These antigen presenting cells (APCs) include B cells, dendritic cells and macrophages.

Cross-presentation

Certain APCs are capable of presenting peptides derived from exogenous proteins upon class I molecules. Such cross-presentation is an important means of priming CD8+ responses in certain viral infections(140, 141).

Antigen specific activation of T cells

The TCR recognises peptides bound to HLA molecules, but the process of T cell activation that follows its binding to antigen is dependent upon other surface proteins, primarily the CD3 complex. CD3 is composed of five transmembrane proteins which contain within their cytoplasmic tails an immunoreceptor tyrosine-based activation motif (ITAM). TCR activation results in phosphorylation of tyrosine residues within the ITAM initiating a cascade of signalling events. Several protein kinases are responsible for the phosphorylation of the ITAM tyrosine(108). CD3 activation also involves ligand interaction with other T cell costimulatory molecules as well as $p56^{lck}$ binding to the cytoplasmic tail of either CD4 or CD8. A simplified overview of T cell activation is given in Figure 16. The CD3 complex alone is capable of bringing about full T cell activation, but this requires ligand triggering of a large number of TCRs(137, 142). T cells become more sensitive to antigenic stimulation if costimulatory receptors are triggered simultaneously and detailed studies of the peptide-class II MHC interaction with a CD4+ T cell demonstrate that even one bound TCR/CD4 is sufficient to trigger calcium influx (a marker of TCR signalling)(143).

Suboptimal CD3 activation in the absence of costimulation results in T cell anergy or apoptosis. TCRs have a relatively low affinity for their peptide-MHC ligands and T cells also bear a range of accessory molecules upon their surfaces which interact with ligands on the cell surface of APCs or target cells to increase the duration of cell-cell adhesion and may enhance signal transduction. Professional APC such as dendritic cells express high levels of costimulatory molecules B7 and CD40, the ligands for CD28 and CD154 respectively. This makes them well placed to stimulate naïve T cells. The need for costimulation provides a safeguard against self-reactivity, the adaptive immune response being dependent in part upon pathogen recognition by innate receptors.

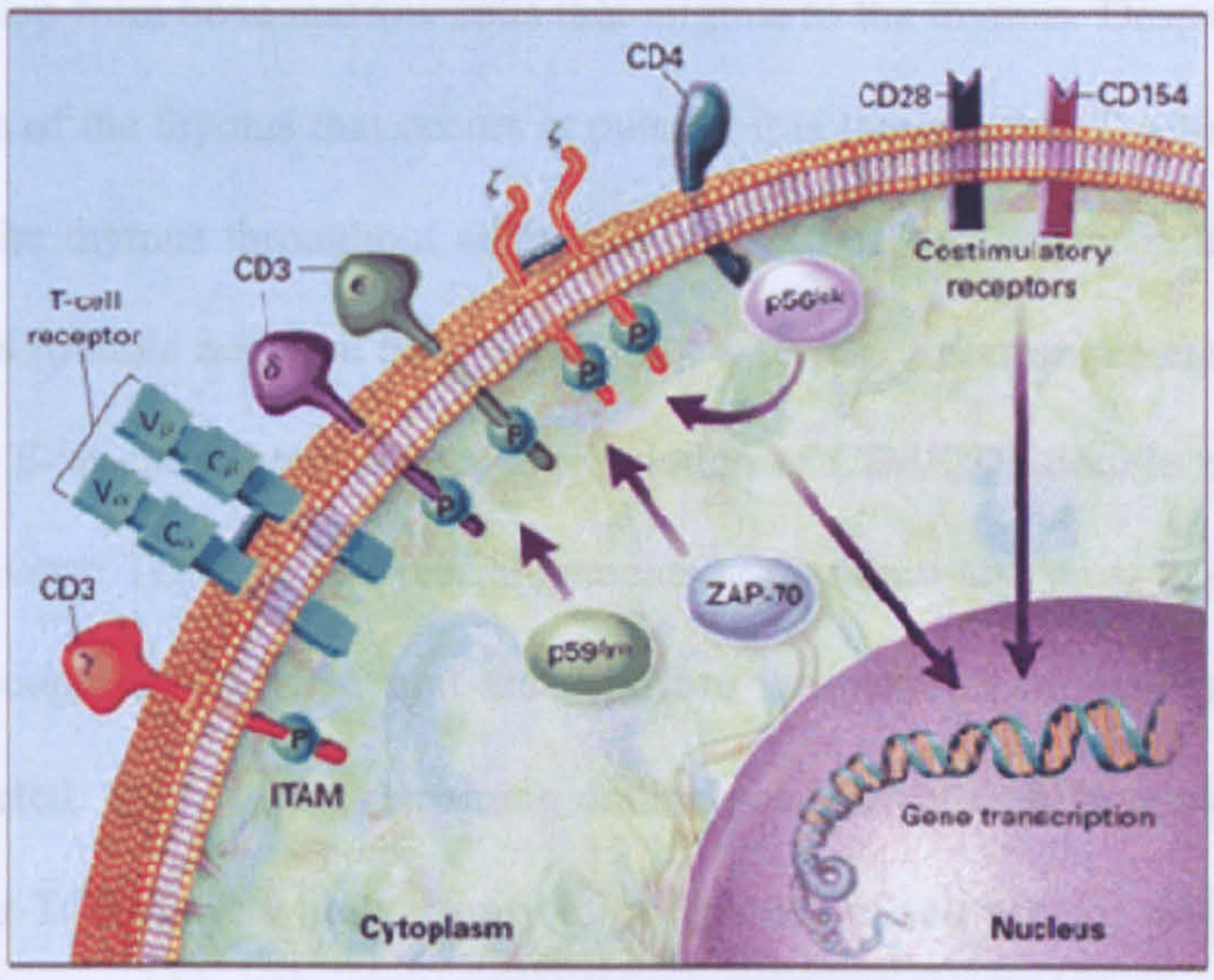


Figure 16. Steps in the activation of T cells. T cell activation involves a complex series of events that follow the cross-linking of TCR on the cell surface. Signalling takes place through proteins associated with the TCR including CD3 and other signal-transduction molecules bearing cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs), which are subject to phosphorylation (P) by protein kinases (e.g. $p56^{lck}$, $p59^{fyn}$, ZAP-70). The initial stages of activation also involve the binding of $p56^{lck}$ to the cytoplasmic tail of CD4 (in helper T cells) or CD8 (in cytotoxic T cells). These events lead to downstream signalling involving a number of different biochemical pathways and ultimately to the transcriptional activation of genes involved in cellular proliferation and differentiation. Signals from costimulatory receptors (e.g. CD28 and CD154) must also be present for activation - signals sent from the TCR signal-transducing molecules alone result in anergy or apoptosis. From Delves PJ, Roitt IM. The immune system. First of two parts. N Engl J Med 2000;343(1):37-49.

CD4 and CD8 coreceptors

CD4 and CD8 are important accessory molecules expressed upon the surface of T cells. They act as HLA coreceptors binding an invariant part of either the class I (CD8) or the class II (CD4) molecule and thus play a part in restricting T helper cells (bearing CD4) to the recognition of peptide-class II HLA molecules and cytotoxic T cells (bearing CD8) to the recognition of peptide-class I HLA molecules. CD4 and CD8 are closely associated with the TCR complex and bind to HLA at the same time as the TCR.

T cell development and selection

T cells develop from bone marrow cells that migrate to the thymus. Despite the partial degeneration of the thymus that occurs at puberty it is thought that T cells continue to develop in the thymus throughout adult life(144, 145). They do not initially express CD4 or CD8 (double negative thymocytes) but a series of rearrangements within the TCR α- and β-receptor genes results a population of CD4/CD8 double positive cells with very diverse TCRs. These rearrangements are random and most thymocytes are unable to recognise self MHC and are therefore incapable of recognising antigen – they are deleted. Many of the remaining double positive cells are potentially harmful because their TCRs have a high affinity for a complex of self peptide and a self MHC molecule (or even an MHC molecule alone). These autoimmune T cells are eliminated by the induction of apoptosis when they interact with dendritic cells and macrophages in the thymic medulla. This leaves T cells with only a weak affinity for self MHC molecules - these cells form the pool of T cells that are exported from the thymus as single-positive CD4 or CD8 cells(108). Less than 1% of all T cell precursors enter the

periphery as naïve T cells. Here they have the potential to recognize a complex of foreign peptide plus self MHC molecules and to become activated if the affinity of the interaction exceeds a certain threshold.

Function of CD4+ T cells

The $CD4^+$ T cell plays a crucial role in protection against viral infection (producing antiviral cytokines as well as providing help to CTL and B cells) and in the development of memory B cells and CTLs. A high affinity interaction between the TCR of a naïve CD4+ T cell and a peptide-class II HLA complex on the surface of an APC leads to IL-2 production and clonal expansion of the T cell(146) – a process that generally takes place within secondary lymphoid organs (e.g. spleen, lymph nodes, Peyer's patches).

- *Cytokine production* – the cytokine milieu in which a naïve CD4+ T helper cell encounters antigen will result in it selecting one of two functional groups. Antigenic stimulation in the presence of IL-12 and IFN-γ will encourage it to adopt the so-called T helper 1 (Th1) phenotype – cells characterised by the production of IFN-γ, TNF-α and IL-2. IL-12 is produced by monocyte-derived DCs – the cell that seems to be the most important in determining the differentiation pathway of the naive T cell(147). Type 1 responses are seen in viral infections, autoimmune disease and antitumour responses(148). One of the primary functions of Th1 $CD4^+$ T cells during a viral infection is the production of cytokines such as IFN-γ and TNF-α which (among others) induce an antiviral state in the host, activate professional APCs for presentation of viral epitopes to $CD8^+$ T cells, and help modulate the humoral and cellular immune response during the course of a viral infection(149, 150). Antigenic stimulation in the

presence of IL-4 induces naïve T cells to adopt a Th2 phenotype, producing cytokines such as IL-4, IL-5 and IL-13 in response to further stimulation(151). Type 2 responses are generally associated with extracellular parasite infections and atopic or allergic disease. The cytokines produced by the Th1 and Th2 subsets both promote differentiation of their own subset and actively inhibit that of the other. It is clear that not all cells fit this paradigm and other subsets described include Th0 cells (those producing mixtures of Th1 and Th2 cytokines and thought to represent "uncommitted" cells with the potential to remain Th0, or differentiate down the Th1 or Th2 route(152)) and even Th3 cells (considered a type of T regulatory cell(153)).

- *Help* – T helper cells activate B cells, driving antibody production down the appropriate route (e.g. Th1 – IgG antibodies, ideal for opsonising bacteria; Th2 – IgM and other isotypes) and Th1 cells in particular activate the microbicidal properties of macrophages.
- *Cytolysis* – although perhaps not their main means of effector function CD4+ T cells are – like CD8+ T cells – capable of directly killing a cell. They do this using mechanisms similar to those employed by CD8+ cells (see below): Fas mediated lysis, direct target lysis and by cytokine release (e.g. TNF-α).

Function of CD8+ cytotoxic T cells

Engagement of the TCR of an antigen-specific CD8+ T cell by the appropriate class I HLA-antigen complex triggers activation of the CD3 complex and the signalling cascade that ultimately results in CTL effector function: direct lysis, Fas-mediated cytotoxicity and noncytotoxic functions.

- *Direct target lysis* – engagement of the TCR leads to lytic granules within the CTL fusing with its surface membrane at the area of target cell apposition. The granule contents are released into the gap and diffuse to the surface of the target cell. One of the constituents, perforin, is functionally very similar to the complement membrane attack complex and integrates into the target cell membrane forming pores. This allows both the entry of another released substance, granzyme B which causes DNA fragmentation, and mechanical cell destruction through osmotic lysis.
- *Fas mediated lysis* - Fas ligand, present on the surface of CD8 CTL, interacts with Fas on the surface of the target cell. This induces a caspase (cysteine aspartic acid protease) cascade which results in genomic DNA fragmentation and the exposure of phagocytosis-stimulating molecules on the cell surface(154).
- *Other effector functions* – CTL also release antiviral cytokines (such as IFN-γ and TNF-α), chemokines (e.g. RANTES, MIP-1α) and other soluble factors. It is the antiviral effect of cytokines over and above direct cell killing that is chiefly responsible for viral clearance in transfer experiments with hepatitis B virus in mice(155). Division of CTL into subsets based upon their cytokine release is less defined than for Th responses but comparable Tc0, Tc1 and Tc2 type responses have been described(156, 157). Tc0 are characterised by the production of IL-4 and IFN-γ (normally associated with either Tc2 or Tc1 phenotypes respectively), Tc1 with IFN-γ, TNF-α and IL-2, and Tc2 with IL-4 and IL-10 among others. Tc0 and Tc2 type cells may have a reduced cytolytic potential and appear to promote Th2 rather than Th1 type CD4+ responses(157).

Immunodominance

For any one virus there are numerous amino-acid sequences capable of binding HLA and the proteasomal processing of all the proteins they express should result in the presentation of an enormous number of foreign peptide-HLA complexes, and the generation of large array of specific CTL. In practice the T cell response is directed against a relatively small number of "immunodominant" epitopes and there is often a clear hierarchy among those responses with certain dominant peptides eliciting stronger responses than other "subdominant" epitopes(158). It is estimated that only 1 in 2000 of all the possible peptides that may be derived from a foreign antigen will become immunodominant for a given class I molecule(159). There are a number of possible explanations for this:

- *The manner in which viral peptides are processed intracellularly* – if a peptide is to be immunogenic it must be processed efficiently and presented on the cell surface. Processing efficiency is affected by the epitope itself(160) and the regions that flank it(161) presumably reflecting the nature of the proteasome's cleaving activity. It might also be expected that epitopes within viral proteins present at high levels in early stages of infection will become immunodominant(162).
- *The affinity of the peptide for the HLA molecule* – compatible peptides from the proteasome become associated with class I HLA within the endoplasmic reticulum. The affinity the peptide demonstrates for the HLA binding site influences immunodominance, probably reflecting the rate of association of a peptide for a given HLA molecule(163).
- *Levels of cell surface expression* – high surface expression of a particular peptide-HLA complex is likely to result from a combination of efficient epitope

processing and a high affinity complex that exhibits a high degree of stability on the surface of the cell(159). Having said that, epitopes expressed at relatively low levels on a cell surface may become immunodominant(164).

- *The TCRs capable of binding the peptide-MHC complex* – the broader the repertoire of naïve T cells that are capable of recognizing a given peptide-HLA complex the more likely it is to become immunodominant(159). Studies have shown that dominant T cell populations express a more diverse TCR repertoire than subdominant T cell populations(165).
- *Suppression of subdominant response by the immunodominant* – the expression of immunodominant epitopes on a cell surface may competitively inhibit the levels of surface expression of subdominant epitopes(166) which may alter the protective efficacy of subdominant responses(167). Blocking processing of dominant epitopes does not necessarily result in the enhancement of previously subdominant responses. It may be that T cells recognizing a dominant epitope expand rapidly, suppressing viral load and suppressing the development of more slowly-generated subdominant responses(158).

The nature of T cell memory

A key feature differentiating the adaptive from the innate immune system is that of pathogen specific memory. It permits a more rapid and effective immune response upon re-challenge by the same (or perhaps in some cases a heterologous) pathogen (168). Memory T cells (CD4+ or CD8+) are derived from cells that have been stimulated by antigen but rather than developing effector functions revert to a quiescent state. Upon antigen re-exposure they are rapidly activated and proliferate producing cytokines in as little as 4 hours(169). Memory populations are generally

considered to fall into two broad classes that reflect the different requirements for rapid and effective recognition of previously encountered pathogens: "central" and "effector" memory. "Central memory" cells express lymph node homing receptors and lack immediate inflammatory or cytotoxic effector function. However from their location within lymphoid tissue they are ideally placed to efficiently stimulate dendritic cells, help B cells and generate a new wave of effector cells upon secondary stimulation. The "effector memory" population comprises tissue-homing cells capable of displaying various immediate effector functions. They represent a readily available pool of antigen-primed cells capable of entering peripheral tissues to mediate inflammatory reactions or cytotoxicity, thus containing invasive pathogens – a immunological rapid reaction force(170). These two memory populations can be distinguished by the presence or absence of CCR7, a chemokine receptor controlling homing to secondary lymphoid organs. Central memory cells are CCR7 positive and express lymph node homing receptors, effector memory cells are CCR7 negative and express receptors mediating migration to inflamed tissues. It has been suggested that this simple two class division is likely to be inadequate(171) particularly in chronic viral infections such as HIV and CMV which lead to the generation of a spectrum of more complex CD8 memory populations.

An acute infection such as influenza infection will result in the activation of a large number of virus-specific effector CTL, a proportion of which will lose activation markers and become quiescent memory lymphocytes. The means by which specific cells are selected for memory is not clear but the TCR repertoires of the primary and memory pools are similar – it is likely that a proportion of the pool of activated lymphocytes is randomly selected for memory(172, 173). The maintenance of effector

CTL is dependent upon antigen. Once the infecting pathogen has successfully eliminated, effector CTL numbers will decline over time by a process of activation-induced cell death (AICD). The precise means by which the memory pool is maintained is controversial. Whether these populations are sustained by continuous low level stimulation by persistent antigen(174) retained within immune complexes on dendritic cells, or memory cells are long-lived and capable of persisting in the absence of antigen(175, 176) has been a matter of some debate. At present at least the balance of opinion would appear to favour the latter hypothesis(100). The size of the memory population that remains following clearance of a viral infection is stable over considerable periods in experimental systems(175). In the real world however the process of homeostasis acting on the memory pool causes "attrition" as a consequence of subsequent, unrelated infections modulating the global memory pool(177, 178). It would appear that memory is a function of both quality and quantity - memory cells have been shown to divide after a shorter lag time and with an increased division rate following antigen stimulation compared to naïve cells. In addition they develop their effector functions faster and are more likely to be multi-functional than primed naïve T cells(179).

No one is naïve – CD8+ T cells and heterologous immunity

The role of antibody in the pathogenesis of severe dengue is widely accepted. Yet there is a growing body of evidence that suggests memory T cell populations may also contribute to the immunopathogenesis of certain severe secondary infections. More than that the T cell memory pool may alter a host's response to infections encountered for the first time.

The TCR recognizes peptide-HLA class I(180) largely through the three protruding CDRs which directly contact sites on the peptide and HLA molecule (Figure 17). If each TCR recognized only a single peptide fragment the number of different T cells required would exceed the number present within an individual. T cells are in fact cross-reactive against many different epitopes and in theory a single TCR may be able to interact with over 1 million different peptides(181). The CDR3 loop is able to adopt different positions to accommodate the varying peptide-HLA structures. This flexibility allows a TCR to show a degree of promiscuity in the spectrum of peptides it can recognize and renders it inherently cross-reactive(182). Thus the cross-reactivity of virus-specific T cells may be a consequence of the similarity between evolutionary conserved sequences between closely related viruses (e.g. influenza subtypes) but need not necessarily be restricted to homologous or highly conserved sequences. In fact memory T cells generated by prior infections may play a role in subsequent infections by both related and unrelated viruses(183). Virus-specific CD8+ T cells have been shown to recognize epitopes from heterologous viruses despite significant sequence variation(184). Individuals exposed to a virus for the first time vary in their symptoms and immune response to infection. These differences are often attributed to the viral dose, or genetic and physiological characteristics of the host. The potentially promiscuous nature of T cell memory suggests another contributor – the varying cross-reactivities of the pool of memory cells specific to previously encountered pathogens.

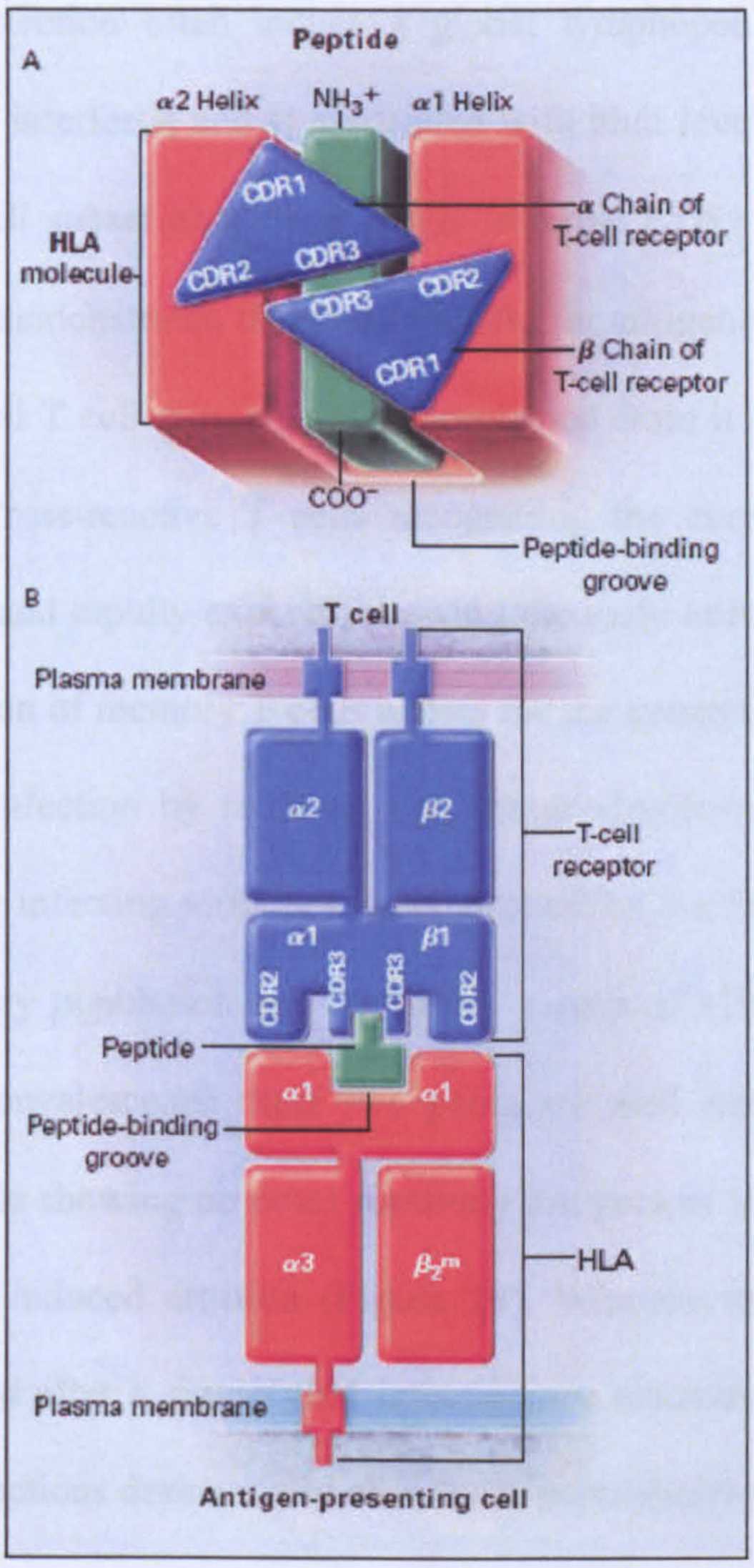


Figure 17. Interactions between the TCR and the peptide-MHC Complex. A) the orientation of the T cell receptor on the surface of the HLA–peptide complex. **B)** the interaction between the T cell receptor and the peptide-MHC complex. Complementarity-determining region 1 of the α and β chains of the TCR is not visible in this depiction because one is positioned behind and the other in front of the part shown. β2-microglobulin (β2m) is the light chain of the class I molecule. The three complementarity-determining regions (CDR1, CDR2, and CDR3) are shown. From Klein, J., and A. Sato. 2000. "The HLA system. First of two parts." N Engl J Med 343:702-709.

The T cell repertoire of a host is significantly altered by each virus it encounters relative to a naïve individual. Populations of memory T cells will have been generated and preserved. Subsequent infections will be impacted by these memory cells, and in turn impact the repertoire of memory that remains once infection is cleared. The initial

stages of a viral infection often induce a global lymphopenia. This cell loss is dependent on type 1 interferon and is associated with high levels of apoptosis in the memory CD8+ T cell subset and lower levels in naïve CD8+ T cells (Figure 18). Mouse studies have demonstrated that cells specific for antigen present at the time of the interferon-induced T cell attrition are not protected from it – all memory T cells are affected(185). Cross-reactive T cells recognizing the currently infecting virus however selectively and rapidly expand following the early attrition phase. It may be that the early depletion of memory T cells allows for the generation of a more diverse T cell response to infection by reducing the immunodomination caused by cross-reactive T cells. The infecting virus is thus controlled by the rapid expansion of the cross-reactive memory population and the newly generated effector T cells specific for that virus. By convalescence these two pools are well represented in memory, whereas memory cells showing no cross reactivity are present in reduced numbers as a result of the IFN induced attrition (Figure 18). Whereas memory CD8+ T cell populations generated after a single viral infection are maintained fairly stably over time, subsequent infections dramatically alter these populations with some non-cross-reactive populations permanently vanishing, and others being enriched by cross-reactive challenge(177, 186). The depletion of components of CD8+ T cell memory may reflect competition for lymphoid space between newly generated memory T cells and existing memory populations, or simply be a consequence of the global lymphopenia induced by viral infection. Either way, pre-existing memory populations are vital to the host response to new viruses encountered and these encounters permanently alter the composition of the memory pool(183).

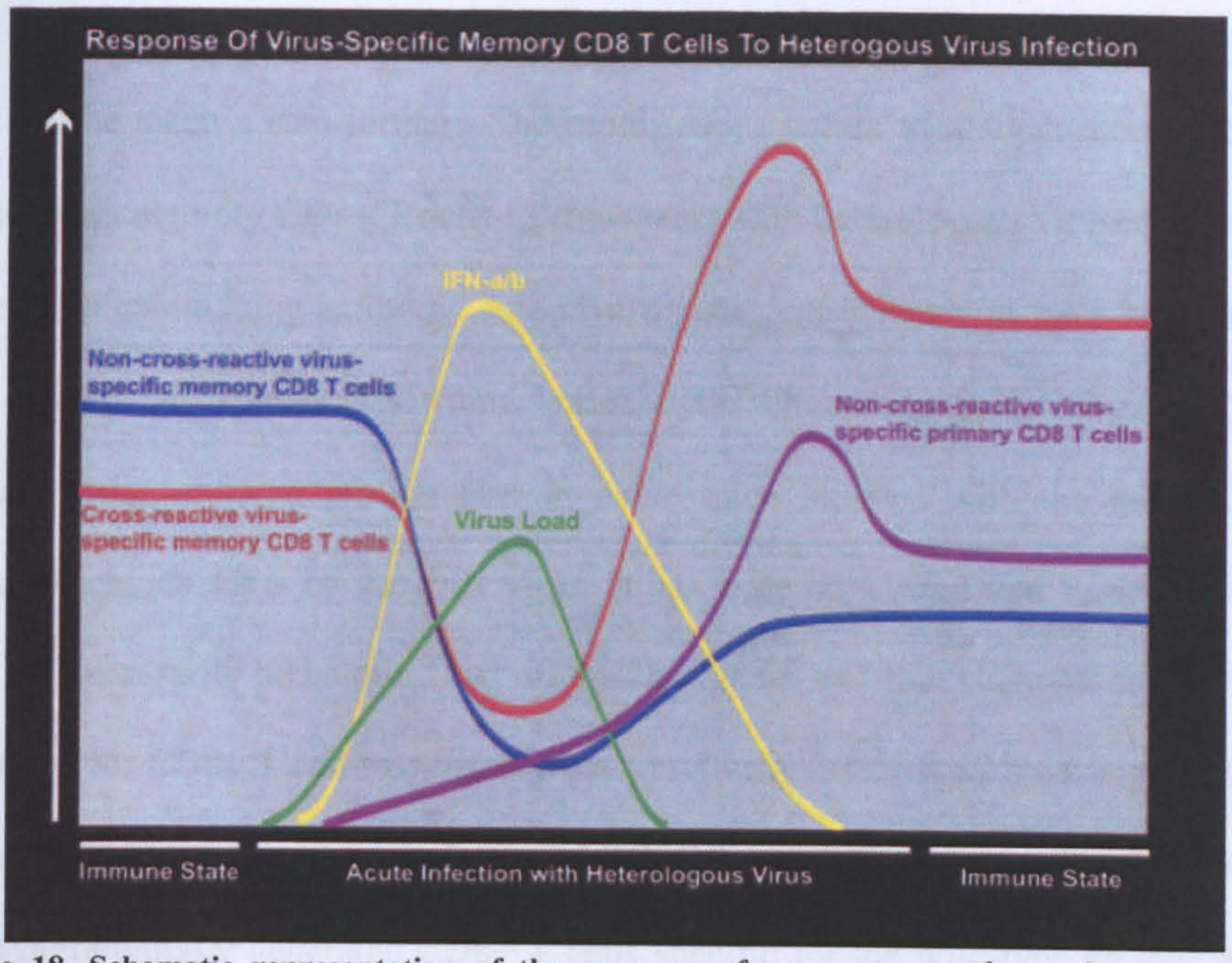


Figure 18. Schematic representation of the response of non-cross-reactive and cross-reactive virus-specific memory CD8 T cells to heterologous virus infection. The X-axis represents time, the Y-axis represents cell number. From Brehm, M.A., L.K. Selin, and R.M. Welsh. 2004. "CD8 T cell responses to viral infections in sequence." Cell Microbiol 6:411-421.

Of course, normally the presence of neutralising antibody will result in a low key secondary T cell response due to the suppression of viral replication and antigen presentation to T cells. An anamnestic T cell response will be mounted in those situations where there is insufficient or absent neutralising antibody, as in the case of infection with a heterologous virus. For example – influenza A virus undergoes antigenic shift which renders antibody to previous forms ineffective. Yet substantial protection remains – this has been attributed to the cross-reactive CD8 T cell response(180). In some cases the outcome may be deleterious to the host. The high level of replication that a heterologous virus is free to do in the absence of neutralising antibody and perhaps even helped by enhancing antibody, might result in a large antigen load. If this is able to activate memory T cells specific for a previously encountered virus profound T cell activation will result(114, 187).

This can be taken a step further – the promiscuous nature of antigen recognition by TCR allows memory CD8+ T cells to cross-react with heterologous viruses for which there is no neutralizing antibody. Adoptive transfer experiments in mice have shown that subsets of splenocytes from lymphocytic choriomeningitis virus (LCMV) immune mice undergo proliferation in naïve mice infected with the heterologous viruses Pichinde virus or vaccinia virus. It has been suggested that these responses may not necessarily be helpful. An "original antigenic sin"-like phenomenon has been described for CD8+ T cell responses. Mice previously infected with wild-type LCMV were challenged with variant strains of LCMV containing altered T cell epitopes. They preferentially generated CD8+ T cells specific for the wild-type epitope which did not efficiently recognize the variants presented by the current infection. This resulted in impaired clearance of the variant virus(188). A similar phenomenon has been described in dengue patients and is discussed further below(189).

The host's history of viral infection may also influence the effectiveness of control of a heterologous virus and even contribute to pathogenesis affecting protective immunity, immunopathology and changing the Tc1/Tc2 balance. Mouse work has shown that initial infection with a given virus can confer partial protection against another *unrelated* virus. This protection is not necessarily reciprocal. For example a mouse previously infected with either LCMV, Pichinde virus or murine cytomegalovirus (MCMV) demonstrated a significant level of protection against infection with vaccinia (reduced viral titres and increased survival). However mice infected with vaccinia did not develop protection against the other 3(190). The lack of reciprocity may relate to the immunodominance of potentially cross-reactive epitopes:

if dominant enough to create a sizeable memory pool protective immunity is conferred. Some authors have hypothesised that large viruses (vaccinia, MCMV) have a better chance of encoding epitopes with the potential to stimulate a cross-reactive response from the memory pool. Small viruses, on the other hand (e.g. Ebola, yellow fever) might escape heterologous memory with the potential for causing rapid and severe disease(191).

Heterologous immunity although potentially protective may be the root of immunopathology as a heterologous virus has the potential to be a strong stimulator of memory T cells specific for another virus because its replication would be unimpeded by neutralising (and perhaps assisted by enhancing) antibodies. LCMV immune mice infected intranasally with vaccinia virus develop severe lung pathology characterised by a strong lymphocytic response with LCMV-specific CD8+ T-cell infiltration and bronchiole obstruction by fibrin and inflammatory cells(192) – bronchiolitis obliterans. This correlated with the activation of pre-existing LCMV-specific memory CD8+ cells. Non-immune mice infected with vaccinia in contrast developed pulmonary oedema. The pathological effect may have been related to altered cytokine profiles – lung lesions in the vaccinia infected LCMV-immune mice were dependent upon the production of IFN-γ.

Whether a T cell adopts a Th1 or Th2 type phenotype is influenced by, among other factors, the concentration of antigen at stimulation and the exposure to cytokines produced by existing Th1 or Th2 cells(193, 194). Thus the Th1 or Th2 bias of a pre-exisiting pool of memory cells activated by heterologous infection might affect the bias of the primary response to that agent. This could explain the high levels of IFN-γ

produced by LCMV-immune mice infected with vaccinia – the original LCMV infection leaves a large Th1 biased memory pool which orients the subsequent response down similar lines(195). Likewise an immunisation that generates a Th2-biased memory response might orient the primary response to a heterologous virus along Th2 lines, impairing viral control and clearance. The inactivated RSV vaccine trialed in the 1960s was associated with severe symptoms following natural infection in a number of recipients. This was associated with pulmonary eosinophilia – a phenomenon known to be associated with a Th2 response. Similar pathology has been replicated experimentally in mouse systems. Mice vaccinated with a recombinant vaccinia/RSV construct develop an aberrant (admittedly CD4+ T cell) Th2 response on RSV challenge that results in eosinophilic lung infiltration(196). Intriguingly mice infected with influenza prior to vaccination and subsequent challenge do not mount such a damaging Th2-type response(197).

The histopathological changes taking place in the lung during acute infection with certain viruses is significantly altered by prior infection with MCMV, influenza and Sendai(198, 199). Certain viral infections cause more significant disease in teenagers than young children (e.g. varicella zoster, measles, mumps). It is possible that pathogens encountered earlier in life alter the immune response to some infections later. Such an argument would lead one to suggest that vaccination could influence the response to pathogens unrelated to the vaccine.

Different epitopes are recognized by the CD8+ T cell population to differing extents – not every recognized epitope will produce equal responses with some mounting only weakly antiviral responses(200). These "immunodominance hierarchies" are reflected in the memory population after acute infection(201). Heterologous infection will alter the spectrum of responses and the hierarchy of immunodominance. Cross-reactive T cells will be selectively amplified by sequential infection by heterologous virus – as has been demonstrated with influenza virus variants(202) – with populations that comprised only a minor part of the memory pool after the original infection becoming more dominant after a second infection. Thus the immunodominance hierarchy of the response to a viral infection is moulded by what has gone before. Figure 19 illustrates this for LCMV and PV – it could equally well be two dengue serotypes.

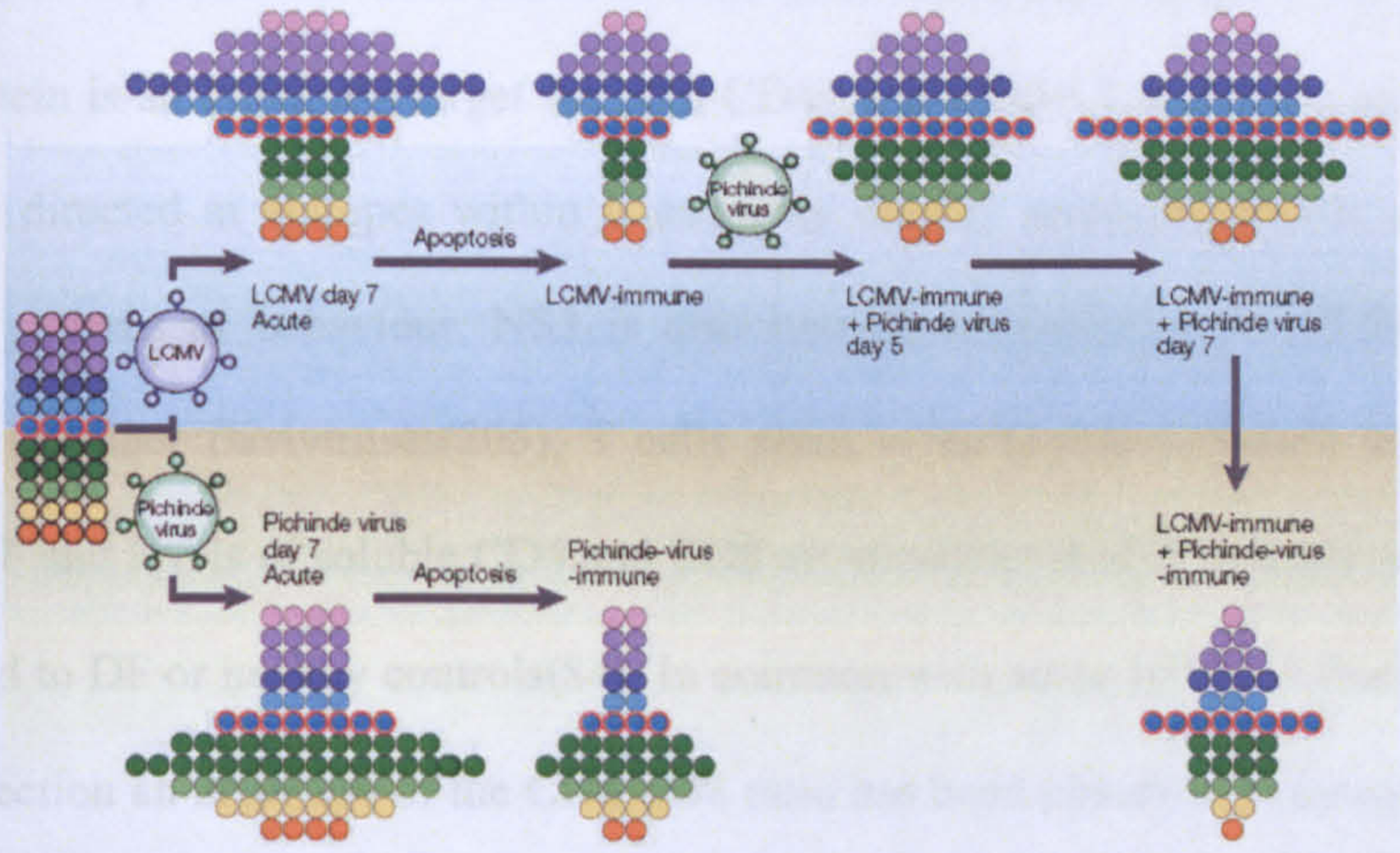


Figure 19. Modulation of the T cell repertoire during viral infection. Dots represent T cell populations of different specificities. A naive immune system is challenged with either LCMV or Pichinde virus (PV). Some T cell populations expand to combat the infection and then undergo apoptosis leaving the host with a skewed memory T cell pool. If an immune system that has been conditioned by one virus infection (LCMV) is exposed to another virus (PV), T cell populations cross-reactive with the two viruses (red outline) expand preferentially and dominate the response. After the response, memory T cells that are specific for the first virus only are reduced in number, whereas the cross-reactive T cells are preserved and enriched in the resting memory pool. From Welsh, R.M., and L.K. Selin. 2002. "No one is naive: the significance of heterologous T cell immunity." Nat Rev Immunol 2:417-426.

The evidence described above relates entirely to studies of CD8+ T cell populations. There is as yet little evidence to suggest that the CD4+ T cell memory populations are modulated by heterologous infections in a similar manner to CD8+ T cells. For example heterologous infections do not accelerate the decline in CD4+ memory T cells in the manner seen with CD8+ T cells(191, 203).

The cellular immune response and dengue pathogenesis

Although much has been hypothesised, little is known about the part cellular immunity might play in either immunity to, or the immunopathogenesis of dengue. Certainly activated T cells can be experimentally infected with dengue virus(204) and dengue virus specific CD4+ and CD8+ memory lymphocytes can be detected in the peripheral blood mononuclear cells of humans experiencing either natural dengue infection or experimental immunisation with live, attenuated dengue vaccines. The NS3 protein is an important target for both CD4+ and CD8+ T cells – many of these cells are directed at epitopes within it and may display serotype-specific or cross-reactive patterns of behaviour. NS3 is also immunodominant in the CD8+ T cell response to other flaviviruses(205). T cells seem to be highly activated in patients with DHF and levels of soluble CD4 and CD8 are most elevated in patients with DHF compared to DF or healthy controls(84). In common with acute HIV and Epstein-Barr virus infection an inversion of the CD4/CD8 ratio has been observed in many patients with acute dengue. CD4+ cells outnumber CD8+ cells in healthy individuals. During acute dengue infection CD8+ cells outnumber CD4+ in some patients, a phenomenon observed more frequently in those with DHF/DSS than DF. Proportions return to normal around 2 weeks after symptom onset(92, 206). It has been suggested that this inversion is an indication of aberrant immune activation.

Interestingly, in terms of *protection* rather than *immunopathology* mouse experiments suggest that it is cytokines that are the key. Mice deficient in their B cell, CD4+ or CD8+ T cells alone had no increased susceptibility to experimental dengue infection. IFN-α/β and IFN-γ receptors on the other hand had critical functions in resolving primary dengue infection and mice deficient in *both* B and T cells were particularly susceptible to infection(106). The dominant role of IFN pathways in controlling viral infection has been observed with other mouse viruses(207).

The role of CD4+ T cells in dengue infection

Infection with one dengue serotype induces both serotype specific and cross-reactive CD4+ T memory cells in most individuals. CD4+ T cell memory is long-lasting, certainly after natural infection. A study in Cuba (where epidemics of DEN-1 then DEN-2 took place 4 years apart with no new outbreaks) demonstrated that individuals who had been infected 20 years previously still had CD4+ responses of both a serotype specific and cross-reactive nature(208).

There is significant complexity in the interactions between T cell receptors and viral epitopes. A given epitope may induce T cells with different cross-reactivities and virus specificities (209, 210) even whilst presented by the same HLA allele(211). Dengue specific CD4+ clones recognise variations of their epitope representing other flaviviruses and may exhibit cytotoxic activity (211), killing antigen-presenting cells primarily by perforin mediated mechanisms (acitivity was blocked by concanamycin) but causing bystander cell lysis by Fas mechanisms(212).

CD4+ cells are an important source of cytokines and dengue specific CD4+ clones may behave in a Th1, Th2 or Th0 manner. They are required for the *in vitro* production of cytokines by PBMCs taken from vaccine recipients. Depleting the CD4+ cells abrogates the dengue-specific cytokine response indicating that either they, or cells stimulated by them, are in turn producing cytokines(213).

The character of the CD4+ T cell cytokine response varies with the nature of the stimulation. Intracellular cytokine staining of PBMC from monovalent vaccine recipients demonstrates that homologous secondary stimulation results in higher frequencies of CD4+ IFN-γ positive cells than stimulation with peptides representing heterologous versions of dengue epitopes(214). One study demonstrated that stimulation with inactivated antigen resulted in IFN-γ production by an average of 0.54%, and TNF-α by 1.7% of CD4+ cells(215). Both were produced at higher levels with homologous stimulation than with antigen from a virus other than the vaccine type. However the ratio of TNF-α to IFN-γ producing cells was higher with heterologous stimulation. This observation was repeated using a pool of class II epitopes from structural and non-structural proteins: TNF-α CD4+ cell responses had broad cross-reactivity for Ag; IFN-γ responses were highest with (and some cases present only in) homologous stimulation. Given IFN-γ's antiviral activity against dengue it could be argued that protection from severe disease in secondary homologous infection requires a healthy IFN-γ response. In contrast TNF-α is produced by memory cells in response to any viral serotype and the immunopathology associated with heterologous secondary infection may be a result of the difference in cytokine balance. It is not clear why different epitopes produce qualitatively different cytokine responses. A quantitative change might be expected if it were simply a

matter of the affinity an epitope-HLA complex displayed for the TCR. The authors of this study speculate that different epitopes may pass through different antigen processing pathways (endogenous or exogenous).

There are other means by which CD4+ cells may affect disease severity. Although most studies have focussed on Th1 type cytokines – those most likely to contribute to a vascular leak syndrome – it has been noted that an early Th1 cytokine profile is replaced by a Th2 pattern at the time of defervescence(92). A recent study noted that the ratio of IFN-γ:IL-4 CD4+ cells on intracellular cytokine staining of ex-vivo PBMC was lower in patients than controls(92). In addition CD4+ cells play a key role in priming naïve CTLs. Their presence or absence can determine whether secondary exposure results in the activation-induced cell death, or the expansion of a CTL population(150).

The role of CD8+ T cells in dengue infection

Mouse models for severe dengue disease have demonstrated that CD8+ cells play both a protective and pathogenic role. 100% of severe combined immunodeficient mice infected with dengue virus die if given naïve thymocytes, compared to 80% given a dengue specific clone. However SCID mice given no CD8+ cells manifest mild symptoms and die later than those with dengue specific CD8+ cells(216). CD8+ cells, more than CD4+ cells, have been shown to infiltrate the liver of mice infected with dengue virus(217).

Cellular immune activation is present early in acute dengue. A study in Thai children showed that absolute CD4+ T cell, CD8+ T cell, NK cell, and gammadelta T cell

counts were decreased in children with DHF compared with those with dengue fever early in the course of illness. The fraction of cells expressing CD69 was increased on CD8+ T cells and NK cells in children who developed DHF more than in those with DF suggesting that CD8+ cell activation may be related to disease severity(218). Severity has been shown to correlate with the frequency of CTL recognising certain dengue epitopes presented by HLA-A*11 and B*07. Patients with DHF have a greater proportion of CD8+ cells recognising those epitopes than those with DF (189, 219).

There is evidence that, as with CD4+ lymphocytes, the nature of the CD8+ memory response to heterologous infection may be different to that seen with secondary homologous infection. One study found that in secondary infection with DEN-1 or DEN-2, a significant portion of dengue specific memory T cells had higher affinity for the DEN-3 form of an A*11 dengue epitope than that of the currently infecting virus(189). The authors called this phenomenon "original antigenic sin", a term first used to describe the persistence of antibody against previously encountered serotypes of influenza(220) and later described as part of the humoral response to dengue(21). They theorise that those CTL exhibiting low affinity for the infecting virus represent a clonal expansion of memory T cells, from a primary infection, which respond more rapidly, to a greater degree and at a lower threshold of activation than do naïve T cells(179). CTL of a higher affinity arise later from the naïve population. This may allow the infection to become established permitting higher viremia and significant immune-mediated tissue damage as T cells die and release cytokines. The number of CTL in DHF patients was significantly lower than that of healthy controls and the majority of dengue specific CD8+ T cells were apoptosing(189). It could be that the higher proportion of low affinity T cells detected were a consequence of the high viral

load driven apoptosis of high affinity T cells via the process of activation-induced cell death (189, 221).

Other reports have also linked cross-reactive cellular immune responses to dengue virus with pathogenesis (222). Serotype cross-reactive memory CTL derived from dengue vaccine recipients have been shown to display a high degree of complexity in their response to heterologous variant peptides. Stimulation with these different peptides produced varying frequencies of CTL producing cytokines in response and the number of different cytokines cells produced. These differences appear to have been affected both by the epitope used, and the vaccination history of the donor(223). These observations give credence to the proposal that cross-reactive T cells might have altered cytokine profiles that could contribute to induction of plasma leakage (222-224).

Vaccine development

The development of a successful dengue vaccine remains the best hope of effective control(60). Vaccines have played a major role in the control of flaviviral diseases such as yellow fever, Japanese encephalitis and tick-borne encephalitis(225). Dengue however remains a significant challenge. The association of DHF with secondary infection has led to legitimate fears of immunisation-mediated disease enhancement(226) – all four serotypes are found in most endemic areas and any vaccine must protect against them all. Several multivalent dengue vaccines are in various stages of development. A live attenuated vaccine is thought by many to be the most likely to succeed. Active intracellular replication results in a variety of antigens that resemble wild-type virus and may provoke a response similar to natural immunity

with strong cytotoxic T cell responses, long-term memory T cells and durable immunity. The replication of each of the four components of a live vaccine (representing the four dengue serotypes) and the immune response to each individual component must not suppress that of the others. As significant cross-reaction between serotypes can be demonstrated for both B-cell and T cell epitopes, this could be difficult to achieve. Any vaccine dependent on stimulating responses to the major virion envelope protein risks the possibility of generating enhancing antibodies unless a complete and balanced immune response to all serotypes can be guaranteed. Even then the kinetics of antibody decline must also be matched to prevent the occurrence of an excess of enhancing antibodies over neutralising antibodies(60). It is rare for an individual to experience more than one or two episodes of severe dengue and it appears that sequential, natural infection results in balanced immunity to more than one virus. However, there is no assurance that artificial simultaneous infection will achieve the same end.

Initial studies of live attenuated tetravalent vaccine produced by serial passage through cell culture have demonstrated that they are capable of producing 80-90% seroconversion rates in both adults and children(227). The use of such vaccines on a large scale in endemic countries would require evidence that they generated solid immunity against all four dengue serotypes with one or two doses. Finding such assurance has been hampered by the absence of an appropriate animal model – mice do not manifest disease and monkeys do not develop the severe illness seen in humans(226). Reversion to virulence through mutation or viral recombination with wild-type virus in an endemic area is also a cause for concern – some authors have called for caution in planning flaviviral vaccine trials because of the risk of

recombination with endemically circulating viruses(228). Other strategies include the use of genetic manipulation techniques to produce infectious clones using a backbone attenuated virus (either the 17D yellow fever vaccine virus or an attenuated dengue virus) into which the preM and E genes of one or more dengue serotypes are inserted(60). In addition dengue genes have been inserted in to plasmids, vaccinia and adenoviral vectors with varying degrees of success(229).

The enhancing antibody response to dengue tends to be directed primarily at the surface proteins. Therefore attempts have been made to overcome ADE by inserting non-structural proteins such as NS1 and NS3 into recombinant virus vectors. They have demonstrated protective immunity in animal systems for other flaviviruses(230, 231). Yet non-structural proteins cannot necessarily be considered entirely innocent in pathogenesis – as we have seen, NS1 and antibodies to it have been associated with pathogenesis.

That these significant problems are only likely to be overcome by international cooperation has been recognized by the establishment of the Paediatric Dengue Vaccine Initiative(232) aimed at funding and conducting research, vaccine development and trials through a network of international partners.

Summary and conclusion

The key features of DHF/DSS are capillary leakage, thrombocytopenia and coagulopathy. The majority of DHF cases occur in patients who have experienced a previous infection with a heterologous DEN serotype. It is associated with immune activation, a high level of cytokine production and appears to induce cross-reactive

anti-dengue antibodies (perhaps directed against NS1) that result in platelet lysis and endothelial cell damage. Dengue virus itself may cause endothelial cell and hepatocyte dysfunction and apoptosis. The rapid resolution of fluid leak seen in those receiving appropriate fluid management would imply that the key feature is not structural damage, but a reversible, probably cytokine mediated, increase in vascular permeability.

The key pathological feature is increased vascular permeability with plasma leakage into the interstitial spaces associated with increased levels of vasoactive cytokines such as TNF-α, IFN-γ, IL-6 and IL-2. A later switch to type 2 cytokine production (e.g. IL-10, IL-13) may occur at the time of defervescence. The levels of certain of these cytokines correlate well with disease severity.

Antibody dependent enhancement of dengue infection by antibodies that neutralise homologous virus but enhance heterologous serotypes has been proposed to be the mechanism behind the phenomenon of severe secondary infection. The increased viral load is thought to result in both direct viral mediated pathology and a deleterious immunopathological response to the increased levels of viral antigen.

ADE certainly appears to take place. In particular it provides a convincing explanation of the phenomenon of infant DHF. However ADE is neither a sufficient (estimates of rates of DHF in those experiencing secondary infection range from 1.8-12%), nor an absolutely necessary (not every severe case occurs in those experiencing secondary infection – although the overwhelming majority do) condition for the development of severe disease. The complications and concerns around vaccine development do not

allow the luxury of resting on one theory of pathogenesis. ADE is unlikely to be the whole story and it is important that a more holistic understanding of DHF pathology is developed, particularly as the need for a safe and effective vaccine becomes ever greater. To assume that the role of cellular immunity is simply to respond to the ADE-augmented viral load overlooks the subtle and complex modulation of the cellular immune response played by T cell memory populations. It has been recognised for some time that CTL populations are capable of mediating significant immunopathology in viral infections such as LCMV. There is good evidence that the CTL response to a viral infection – whether heterologous or unrelated to previous viral encounters – can be modulated by the infection history of an individual in a manner likely to contribute to disease severity. With four stable heterologous serotypes dengue virus is highly likely to participate in just such a process. It is to these questions of cross-reactive T cell responses that this thesis now turns, in particular the manner in which CD4+ and CD8+ T-memory cells showing dengue virus, or perhaps even broader flaviviral, cross-reactivity might contribute to both protection from, and the development of, severe dengue disease.

CHAPTER 2 MATERIALS AND METHODS

Study population and ethical approval

Blood samples were collected from a cohort of adults and children enrolled in a prospective study of dengue virus infection at The Hospital for Tropical Diseases (HTD) and Ho Chi Minh City Children's Hospital #1, Ho Chi Minh City, Viet Nam. Both serve the local community and act as tertiary referral hospitals for patients in southern Viet Nam. Patients were recruited if there was a clinical suspicion of dengue virus infection and the diagnosis confirmed by serological and virological assays. Each patient's illness severity was classified according to World Health Organisation criteria(14) after a review of the study notes. Samples were collected on the day of admission (acute), on the third and fifth day if the patient remained in hospital, and during convalescence (two weeks to one month after the first sample). Patients were characterised demographically, clinically, virologically (using RT-PCR) and serologically (IgM and IgG on paired samples). Serological data suggested that the majority (>99%) of the patients we studied were experiencing secondary infections. Informed consent was obtained from all participants at enrolment and the study protocol was approved by the Scientific and Ethics committee at The Hospital for Tropical Diseases and the Oxford Tropical Research Ethics committee.

Solutions and reagents

Supplemented RPMI-1640 complete tissue culture media "R10"and "H10".

R10 - RPMI (Gibco, Paisley UK) was supplemented with L-glutamine 100U/ml, penicillin 100U/ml, streptomycin 100µg/ml (all Gibco, Paisley, UK) and heat inactivated foetal calf serum 10% (Labtech Intl, UK). Stored at 4°C.

H10 – as above but rather than foetal calf serum (FCS) the solution was supplemented with 10% heat inactivated human serum pooled from at least 2 blood group AB positive donors.

Freezing media

FCS (filtered through a sterile 0.22μm filter – Millipore) with 10% dimethlyl sulphoxide (DMSO, Sigma). Stored at 4°C. Cell lines and PBMC for storage were pelleted, resuspended in 1ml of cold freezing media and placed in a controlled cooling box (isopropanol based). This was stored at -80°C and cells transferred to liquid nitrogen storage after a few days.

Phosphate buffered saline (PBS)

Salts were supplied by Gibco, Paisley, UK and dissolved in filtered, deionised water. The solution was autoclaved to ensure sterility and stored at room temperature (RT).

Alkaline phosphatise-conjugate substrate kit (chromogen for ELIspot)

From Bio-Rad Laboratories, USA. Development solution made by mixing 0.4ml development buffer, 9.6ml deionised water, 0.1ml colour reagent A, 0.1ml colour reagent B. Applied 100μl/well.

Phytohaemagglutinin (Murex Biotech Ltd, UK)

Made to a stock solution of 10mg/ml in sterile water. Used as a positive control in ELIspots and cytokine stimulations at 0.1μl per 100μl culture media.

Heparin sodium

Obtained from CP pharmaceuticals, Wrexham, UK at 5000 units/ml. Stored at room temperature.

Trypan blue

From Sigma, UK. Diluted and used to assess cell viability and facilitate cell counting when required. Stored at room temperature.

FACS wash

100ml PBS, 1ml FCS, 0.001% sodium azide. Stored at 4 °C.

Ampicillin

1000x ampicillin stock made by dissolving 100mg/ml ampicillin in sterile water and passing through a 0.22μm sterile syringe filter (Millipore). Kept at -20°C.

LB media

10g typtone, 5g yeast extract, 10g NaCl (or 5g NaCl in low salt LB media) made to 1 litre in distilled water and autoclaved. 1ml of ampicillin stock solution was added to each litre before use.

Agar plates

15g of agar powder was dissolved in 1 litre of LB which was then autoclaved. 1ml of the 1000x stock ampicillin solution was added once the solution had cooled below around 50°C. The warm agar was then poured into Petri dishes and allowed to set in a sterile tissue culture hood.

IPTG (isopropyl thiogalactoside)

1 Molar stock was made by dissolving 2.38g of IPTG powder in 10mls of ddH_2O. This was filtered through a 0.22μm syringe filter and frozen until use.

X-Gal (5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside)

X-Gal powder was dissolved in dimethyl formamide at 20mg/ml and frozen until use.

TBE (Tris-borate-EDTA) buffer

5 x stock: 5.4g Tris, 2.75g borate, 0.372g EDTA in 100ml of ddH_2O.

Electrophoresis gel (0.8% agarose)

0.48g of electrophoresis grade agarose powdered added to 60ml of 0.5 TBE buffer and heated until fully dissolved. Ethidium bromide (Sigma) was added just before pouring the cooling solution into the gel tank.

Triton wash buffer

1ml Triton X-100, 10ml 1M Tris pH8, 20ml 1M NaCl, 2ml 10% sodium azide, 400μl of 0.5M EDTA, 100μl of 2M DTT, made up to 200ml in ddH_2O.

Triton free wash buffer

As above but without the 1ml Triton X-100.

Tetramer refold buffer

100ml of 1M Tris pH8, 84.28g L-arginine, 4ml 0.5M EDTA, 1.54g reduced glutathione, 0.31g oxidised glutathione, 1ml of 0.1M phenylmethyl-sulphonylfluoride (PMSF – a serine protease inhibitor), made to 1 litre with ddH_2O.

Urea-based protein solubilisation solution

40ml 10M urea, 5ml 1M NaH_2PO_4, 250µl 2M Tris pH8, 10µl 0.5 M EDTA, 2.2µl 2.2M DTT.

Specimen acquisition

Peripheral blood samples were collected by ward staff using heparin sodium as an anticoagulant. 1-2ml of blood was taken from children, up to 10ml from adults. Samples were generally transported to the laboratory within 2 hours – occasional samples were delayed by up to 10 hours. Samples were centrifuged and the serum fraction removed for freezing. Peripheral blood mononuclear cells (PBMC) were then isolated by Ficoll-Hypaque density gradient centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway). After washing they were either cryopreserved in freezing medium or resuspended in R10 used immediately. Certain of the cryopreserved specimens were shipped on dry-ice to Oxford.

HLA Typing

Molecular HLA typing was performed on most study subjects by a laboratory technician in Oxford using amplification refractory mutation system PCR (ARMS-PCR) with sequence specific primers, as previously described (233). A commercial

typing application, the Dynal RELI SSO HLA typing kit (Dynal Biotech, United Kingdom) was used in Viet Nam to type samples from some patients.

Viral identification by reverse-transcription PCR

Dengue virus RNA in acute plasma samples was isolated with RNAgents (Promega, Madison, Wisconsin). RNA was reverse transcribed and two rounds of PCR performed using primers and methods previously described(234). In samples containing virus, the PCR yielded DNA products of size uniquely characteristic of each dengue virus serotype.

Serological testing

Dengue virus infection was confirmed by serological testing of acute and early convalescent-phase plasma samples taken at least 3 days apart using a commercial capture-immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) system (Panbio, Brisbane, Australia). Results were interpreted according to the manufacturer's instructions. The ELISA assay has been validated as sensitive and specific for primary and secondary dengue virus infection(235). The term "secondary infection" describes the nature of the serological response – one cannot necessarily conclude that the patient was experiencing only their second dengue infection.

Synthesis of dengue NS3 peptides

The dengue non-structural protein NS3 contains many T cell epitopes(219). Using the consensus sequence for each dengue serotype (1-4) overlapping peptides spanning the

NS3 protein were synthesised using standard, solid-phase 9-fluorenylmethoxy carbonyl chemistry. Each was 15 amino acids long and overlapped the next by 10 amino acids producing 124 peptides for each serotype. The peptides for dengue 2 (strain 16681) were produced in Oxford using a Zinnser Analytical synthesiser (Advanced Chemtech, Louisville, KY) – this apparatus was also used to produce other peptides as required, e.g. for elucidating a minimal epitope. Those for serotype 1, 3 and 4 were produced commercially (Hybio, China). Purity was established by high-pressure liquid chromatography (HPLC) and ranged from 30 to 90%. Each peptide was dissolved in DMSO (Sigma) and then made up to a concentration of 2mg/ml in RPMI. The final DMSO concentration was never over 4%. The amino acid sequences for the 4 NS3 variants can be found in appendix 1.

Interferon gamma (IFN-γ) ELISpot assays with PBMC

An ELISpot assay was used to detect peptide-specific IFN-γ release by PBMC. 96-well polyvinylidene difluoride-backed plates (MAIP45; Millipore, Massachusetts, USA) were precoated with 15μg of anti-IFN-γ monoclonal antibody 1-DIK (Mabtech AB, Nacka, Sweden) per ml for 2 hours at 37°C. The wells were washed 3 times with phosphate-buffered saline (PBS) with 0.05% Tween-20 (Sigma) and excess protein binding sites on the membrane blocked by coating with R10 or H10 and leaving at room temperature for 1 hour. 100,000 PBMC were incubated overnight in 100μL of either H10 or R10 at 37°C in 5% CO_2 in duplicate wells with either: peptide at a concentration of 0.1 to 10μM; R10 or H10 alone (negative control); 1:100 phytohaemagglutinin (PHA) (Murex Biotech Ltd, Dartford, UK) as a positive control. Cells were discarded and the plate washed with PBS/Tween before incubating at RT

for 3 hours with a biotinylated anti-IFN-γ monoclonal (7-B6-1 biotin; Mabtech) at a concentration of 1μg/ml. The plate was washed with PBS/Tween and incubated at room temperature with streptavidin-conjugated alkaline phosphatase (Mabtech) at a dilution of 1:1000 PBS for 1-2 hours. IFN-γ producing cells could be detected as dark spots using the alkaline phosphatase-conjugate substrate kit prepared as described above. Spots were counted using an automated ELISpot reader (AID, Sweden). The background (negative control) was subtracted from each well. PBMC stimulated with PHA served as a positive control. The response from a well was considered positive if its corrected count was twice that of the background.

Interferon gamma (IFN-γ) ELISpot assays with T cell lines and clones.

Clones and lines were tested for specificity in a cultured ELIspot assay. The plate was prepared as described above. HLA-matched B cell lines were pulsed with peptide at the desired concentration for 1 hour at 37°C and washed. Cells were distributed between wells such that each contained 5000 B cells and 500 of the T cell clone or line. Unpulsed B cells were used as a negative control. They were incubated overnight at 37°C and then developed as described above.

Establishment of CTL lines and clones

CTL lines were generated as previously described (236). PBMCs were stimulated with the specific epitope peptide of interest at 2μM concentration. IL-2 was added on day 3, and the specificity of the CTL lines were tested using CTL lysis assays or

tetramer staining on day 10 and/or day 20. Cloning mix was prepared by taking the irradiated freshly separated PBMCs of at least 3 donors and resuspending them in H10 at a concentration of 1 million cells per ml. PHA was added to this to a final concentration of 0.033mg/ml and the suspension mixed well. CTL clones were established from PBMCs or CTL lines by one of two methods.

1. Where the appropriate antigen-specific tetramer was available cells could be stained with tetramer and CD8 fluorescent antibody and double positive cells sorted by flow cytometric sorting directly into 100μL of cloning mix in a 96 well round-bottomed plate.
2. Alternatively antigen-specific cells were enriched using MACS magnetic microbeads (Miltenyi Biotec, Germany). These were used in one of three ways:
    a. Cells were stimulated for 4 hours in 96 well plates with the peptide of interest and then labelled with a proprietary IFN-γ catch reagent to mark those cells that were producing IFN-γ in response to the stimulation. They were then labelled with an IFN-γ detection antibody and magnetic beads.
    b. Anti-CD8 microbeads were used to simply enrich the CD8+ T cell fraction.
    c. Cells were stainined with PE-conjugated tetramer and MACS anti-PE-microbeads used to select tetramer positive cells. Briefly tetramers were added to 3-5 million PBMCs, then incubated at 37°C for 20 minutes. The cells were washed with cold buffer and resuspended in 40μl of anti-PE beads mixed well and incubated for 15 minutes at 4-8°C shaking every 5 minutes.

Once cells were labelled with the magnetic beads they were washed, resuspended in 500µl of cold FACS buffer and magnetic separation performed according to the manufacturer's protocol (Miltenyi Biotec, Germany). Cells were resuspended in H10 and counted. A proportion of these were added to cloning mix which was in turn distributed at 100µL per well throughout a 96 well round bottomed plate at an average of 0.3 cells per well.

The number of plates required varied with the anticipated frequency of antigen-specific cells. Where cells were sorted by flow-cytometry 1-5 plates were sufficient. Where enrichment was solely by CD8 beads 10 or more plates were prepared. IL-2 was added to wells at day 3 (200 units/ml). At around 2-3 weeks those wells with growing cells were tested for specificity using tetramer staining or cultured ELIspot. Epitope specific cells were expanded and maintained by periodic restimulations using the cloning mix described above, moving into larger plates or small flasks as appropriate.

CTL lysis assays

CTL lysis assays were performed using standard ^{51}Chromium release assays (236). HLA class I matched B-cell lines (BCL) were pelleted and incubated with 7.4 MBq of ^{51}Chromium (around 200µl of stock solution – Amersham Ltd, UK) for 1 hour. After washing three times target cells were then divided and pulsed with either no peptide (negative control) or peptides at different concentrations. After another hour of incubation at 37°C, the peptide solution was then washed off and cells were counted

and co-cultured with CTL clones at appropriate effector to target (E: T) ratios in 96 well plates. The plates were incubated at 37°C for 4 hours after which 30µl of the supernatant from each well was transferred into 150µl of Optiphase Supermix in a 96 well reading plate (Wallac, Finland). Radioactivity was counted using a Beta-plate counter (Wallac). Specific lysis was calculated from the formula:

% lysis =(experimental counts - media control) / (detergent control- media control) x 100 %.

Cytokine beads assay

Cytokine production by clones was measured using a protocol similar to that above but with unlabelled B cells. HLA class I matched BCL were washed and pulsed with either no peptide (negative control) or peptides at different concentrations for 1 hour at 37°C. After washing and counting, cells were co-cultured with CTL clones at appropriate effector to target ratios in round-bottomed 96 well plates overnight in a total volume of 150µl of H10. 70µl of supernatant was then harvested and either used in assays immediately or frozen at -80°C. Cytokines were measured by Luminex cytokine bead array analysis according to the manufacturer's instructions (Bio-Rad Laboratories, USA).

Antibodies

Antibody	*Label*	*Company*
Anti-CD4	PE, Pacific Blue	BD biosciences, USA
Anti-CD8	PerCP, APC, PE-Cy7	BD biosciences, USA
Anti-CD38	FITC	BD biosciences, USA
Anti-CD107a/b	FITC	BD biosciences, USA
Anti-IFN-γ	APC	Serotec, USA
DK25 CD8 blocking	None	DAKO, USA.

Cell surface staining

Cell surface staining was carried out on freshly separated or carefully thawed cryo-preserved PBMCs. Titrated tetramers conjugated to either PE (Sigma, UK), APC (Molecular Probes) or quantum-red (Sigma) were added for 15 min at 37°C. Cells were incubated with CD8-APC and CD38 FITC antibodies for 15 min at RT in some cases. Cells were then washed and stored in Cell Fix™ buffer (Becton Dickinson) at 4°C until flow cytometry analysis was performed. Samples were analyzed on a Becton Dickinson FACSCalibur.

Cell surface and intracellular cytokine staining

Intracellular cytokine staining was performed on cell lines, clones or PBMC that had undergone stimulation with either peptide or peptide-pulsed B cells for a period of time. Where B-cells were used to present antigen they were pulsed with peptide or negative control (RPMI) for 1 hour at 37°C and then incubated with the effector cell

at a ratio of 1:1 (usually around 50000 to 100000 cells each) in 96 well round bottomed plates at 37°C in 200µl H10. In all cases 1µl of monensin (eBioscience, USA) was added to each well after 1 hour to halt the export of cytokines to the cell surface. Incubation continued for a further 4 hours and cells then washed in FACS wash. Titrated tetramers (PE, APC or quantum-red conjugated) were added for 15 min at 37°C if required. Cells were then incubated with CD8-APC, CD38 FITC or other surface antibodies as required for 15 min at room temperature (RT). Cells were washed in FACS wash twice, resuspended in 100µl of permeabilisation solution (BD CytoFix/CytoPerm) and kept at 4°C for 30 minutes. They were then washed in Perm Buffer twice and resuspended in 100µl of Perm Buffer. Intracellular staining antibodies were added and the mixture kept in the dark at RT for 20 minutes. Cells were washed twice with Perm Buffer and then resuspended in 200µl of Perm Buffer and kept in the dark at 4°C until analysis. Samples were analyzed on either a Becton Dickinson FACSCalibur, or Dako CyanADP.

CD107a/b staining of clones

This was performed as previously described(237). Peptide pulsed/unpulsed B cells were incubated with T cell clones as described above with the addition of 2µl of both CD107a FITC and CD107b FITC to each well. This is necessary due to the transient expression of CD107a/b after stimulation. Monensin was added at 1 hour – the remainder of the protocol is identical to that described above.

TCR Vβ antibody staining

Approximately 50000 cells of a T cell clone were placed in a V-bottomed 96 well plate and pelleted by centrifugation. The supernatant was pipetted off and 2μl of the appropriate human Vβ antibody (raised in mice – Immunotech, France) added to the cells. After 20 minutes at room temperature the cells were washed twice with FACS buffer and the supernatant pipetted off. 5μl of a 1 in 20 PBS dilution of rabbit-anti-mouse-IgG FITC antibody (BD biosciences, USA) was added and the cells left in the dark for 20 minutes at room temperature before washing twice in FACS buffer. Cells were then fixed and analysed as above.

Tetramer decay assay

Tetramer decay assays give an indication of the relative avidity of two or more clones for a given tetramer. Clones are stained with tetramer but rather than fixing are then incubated with an excess of a competing ligand – either the same tetramer bound to a different marker (e.g. APC)(238) or an antibody capable of blocking tetramer rebinding. As the original tetramer vacates the TCR its place is taken by the alternative ligand and it cannot rebind. Comparing the fall in fluorescence intensity between clones allows a crude assessment of relative avidity – the faster the fall, the lower the avidity. Such assays have been found to correlate with functional avidity measured by such surrogates as cytolytic function(238). Cells from a CTL clone were incubated with PE-labelled tetramer for 40 minutes at 4°C. The cells were washed twice and resuspended in 50μL of buffer. 2μL of this suspension was added to 100μL of PBS and analysed by flow cytometry. Either a 5-10 times excess of identical tetramer conjugated to a different label, or an excess of antibody known to block

tetramer binding (DK25, Dako) was then added to the remaining cell suspension. 2μL of this reaction was removed periodically, added to 200μL of PBS and analysed on a Cyan ADP flow cytometer. The fraction of positive cells was defined as the percentage of cells falling above a gate at which 90% of cells were positive at time 0.

CHAPTER 3 PRODUCTION OF NOVEL HLA CLASS I TETRAMERS

Introduction

The development of tetrameric synthetic HLA-peptide complexes revolutionised the study of certain aspects of immunology(239). Tetramers consist of 4 synthetic HLA molecules - monomers. Each monomer consists of a synthetic heavy chain and β2-microglobulin complexed with the peptide antigen of interest. Heavy chains are derived from cloned wild-type cDNA modified by substitution of the transmembrane and cytosolic regions with a sequence encoding the BirA biotinylation enzyme recognition site, as previously described (240). Biotin is added at this point using the BirA enzyme. The fluorochrome used to label the monomer has 4 biotin binding sites thus allowing it to bind 4 monomers when conjugated: the tetramer. Their development has allowed the detection, enumeration, collection and phenotypic study of cells recognising specific antigens.

Just as *in vivo* the HLA molecule is bound by both the TCR and the CD4 or CD8 molecule, so too are tetramers when staining a cell that recognises them. It has been demonstrated that modification of the HLA molecule's CD8 binding region in a manner that abrogates CD8 binding allows the generation of tetramers that bind only cells demonstrating a high affinity interaction at the TCR(241, 242). This chapter describes the generation of novel tetramers – both those based upon the wild-type HLA sequence and those modified in their CD8 binding regions for the purpose of detecting high affinity antigen specific cells recognising previously described

epitopes. These epitopes are located within NS3, restricted by A*11(189) and B*07(187). The A*11 epitope differed between DEN-1 (GTSGSPIINR) and DEN-2 (GTSGSPIIDK), with DEN-3 and -4 possessing identical sequences (GTSGSPIVNR). The B*07 epitope differed between DEN-1 (APTRVVASEM) and DEN-2, 3 and 4 (APTRVVAAEM).

Method

Modification of existing constructs by site-directed mutagenesis

Existing plasmids containing the sequence for the heavy chain monomers of B*070201, B*070501 and A*110101 were used as templates, modified to produce monomers of B*070501, B*070501 CD8 non-binding and A*110101 CD8 non-binding respectively. The original sequences and sites of the changes required are illustrated in Figure 20, Figure 21 and Figure 22. The existing B*0702 tetramer did not work well with samples from Viet Nam. This was thought to be due to the commonest Vietnamese subtype being B*0705. The alignment of these 2 subtypes (Figure 20) illustrates a single difference – the aspartic acid of 0702 is an asparagine in 0705. This occurs in a key binding region. Making this single amino acid/nucleotide change would increase the chances of detecting antigen specific cells. Producing CD8-non-binding tetramers for both A*1101 and B*0705 required the introduction of a mutation in the region of amino acids 227 and 228 (the alpha-3 domain of the MHC molecule) as illustrated in Figure 21 and Figure 22. Changing the DT (aspartic acid, threonine) found here to KA (lysine, alanine) has been shown to eliminate CD8 binding to the HLA molecule(241) and such tetramers reliably detect high avidity cells(242).

```
                    -21       -11       -1        10        20        30        40        50        60        70
B*070201            MLVM APRTVLLLLS AALALTETWA GSHSMRYFYT SVSRPGRGEP RFISVGYVDD TQFVRFDSDA ASPREEPRAP WIEQEGPEYW DRNTQIYKAQ
B*070501            ---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                     80        90       100       110       120       130       140       150       160       170
B*070201            AQTDRESLRN LRGYYNQSEA GSHTLQSMYG CDVGPDGRLL RGHDQYAYDG KDYIALNEDL RSWTAADTAA QITQRKWEAA REAEQRRAYL EGECVEWLRR
B*070501            ---------- ---------- ---------- ---------- ---N------ ---------- ---------- ---------- ---------- ----------
                    180       190       200       210       220       230       240       250       260       270
B*070201            YLENGKDKLE RADPPKTHVT HHPISDHEAT LRCWALGFYP AEITLTWQRD GEDQTQDTEL VETRPAGDRT FQKWAAVVVP SGEEQRYTCH VQHEGLPKPL
B*070501            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    280       290       300       310       320       330       340
B*070201            TLRWEPSSQS TVPIVGIVAG LAVLAVVVIG AVVAAVMCRR KSSGGKGGSY SQAACSDSAQ GSDVSLTA
B*070501            ---------- -I-------- ---------- ---------- ---------- ---------- --------
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                                -20                 -15                 -10                  -5                   1
B*070201            ATG CTG GTC ATG GCG CCC CGA ACC GTC CTC CTG CTG CTC TCG GCG GCC CTG GCC CTG ACC GAG ACC TGG GCC G|GC
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -|--
                                 5                   10                  15                  20                  25
B*070201            TCC CAC TCC ATG AGG TAT TTC TAC ACC TCC GTG TCC CGG CCC GGC CGC GGG GAG CCC CGC TTC ATC TCA GTG GGC
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                30                  35                  40                  45                  50
B*070201            TAC GTG GAC GAC ACC CAG TTC GTG AGG TTC GAC AGC GAC GCC GCG AGT CCG AGA GAG GAG CCG CGG GCG CCG TGG
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                55                  60                  65                  70                  75
B*070201            ATA GAG CAG GAG GGG CCG GAG TAT TGG GAC CGG AAC ACA CAG ATC TAC AAG GCC CAG GCA CAG ACT GAC CGA GAG
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                80                  85                  90                  95                 100
B*070201            AGC CTG CGG AAC CTG CGC GGC TAC TAC AAC CAG AGC GAG GCC G|GG TCT CAC ACC CTC CAG AGC ATG TAC GGC TGC
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- -|-- --- --- --- --- --- --- --- --- ---
                               105                 110                 115                 120                 125
B*070201            GAC GTG GGG CCG GAC GGG CGC CTC CTC CGC GGG CAT GAC CAG TAC GCC TAC GAC GGC AAG GAT TAC ATC GCC CTG
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- ---
                               130                 135                 140                 145                 150
B*070201            AAC GAG GAC CTG CGC TCC TGG ACC GCC GCG GAC ACG GCG GCT CAG ATC ACC CAG CGC AAG TGG GAG GCG GCC CGT
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               155                 160                 165                 170                 175
B*070201            GAG GCG GAG CAG CGG AGA GCC TAC CTG GAG GGC GAG TGC GTG GAC TGG CTC CGC AGA TAC CTG GAG AAC GGG AAG
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               180                 185                 190                 195                 200
B*070201            GAC AAG CTG GAG CGC GCT G|AC CCC CCA AAG ACA CAC GTG ACC CAC CAC CCC ATC TCT GAC CAT GAG GCC ACC CTG
B*070501            --- --- --- --- --- --- -|-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               205                 210                 215                 220                 225
B*070201            AGG TGC TGG GCC CTG GGT TTC TAC CCT GCG GAG ATC ACA CTG ACC TGG CAG CGG GAT GGC GAG GAC CAA ACT CAG
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               230                 235                 240                 245                 250
B*070201            GAC ACT GAG CTT GTG GAG ACC AGA CCA GCA GGA GAT AGA ACC TTC CAG AAG TGG GCA GCT GTG GTG GTG CCT TCT
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               255                 260                 265                 270                 275
B*070201            GGA GAA GAG CAG AGA TAC ACA TGC CAT GTA CAG CAT GAG GGG CTG CCG AAG CCC CTC ACC CTG AGA TGG G|AG CCG
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -|-- ---
                               280                 285                 290                 295                 300
B*070201            TCT TCC CAG TCC ACC GTC CCC ATC GTG GGC ATT GTT GCT GGC CTG GCT GTC CTA GCA GTT GTG GTC ATC GGA GCT
B*070501            --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               305                 310                 315                 320                 325
B*070201            GTG GTC GCT GCT GTG ATG TGT AGG AGG AAG AGT TCA G|GT GGA AAA GGA GGG AGC TAC TCT CAG GCT GCG T|GC AGC
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- -|-- --- --- --- --- --- --- --- --- --- --- -|-- ---

                               330                 335                 340
B*070201            GAC AGT GCC CAG GGC TCT GAT GTG TCT CTC ACA GCT TGA
B*070501            --- --- --- --- --- --- --- --- --- --- --- --- ---
```

Figure 20. Alignment of B*070201 and B*070501 amino acid (upper panel) and nucleotide (lower panel) sequences. The change required to produce the latter from the former is indicated in red. Sequences from the IMGT/HLA database.

```
                    -21        -11         -1          10         20         30         40         50         60         70
A*110101          MAVM APRTLLLLLS GALALTQTWA GSHSMRYFYT SVSRPGRGEP RFIAVGYVDD TQFVRFDSDA ASQRMEPRAP WIEQEGPEYW DQETRNVKAQ

                   80         90        100        110        120        130        140        150        160        170
A*110101  SQTDRVDLGT LRGYYNQSED GSHTIQIMYG CDVGPDGRFL RGYRQDAYDG KDYIALNEDL RSWTAADMAA QITKRKWEAA HAAEQQRAYL EGRCVEWLRR

                  180        190        200        210        220        230        240        250        260        270
A*110101  YLENGKETLQ RTDPPKTHMT HHPISDHEAT LRCWALGFYP AEITLTWQRD GEDQTQDTEL VETRPAGDGT FQKWAAVVVP SGEEQRYTCH VQHEGLPKPL

                  280        290        300        310        320        330        340        350
A*110101  TLRWELSSQP TIPIVGIIAG LVLLGAVITG AVVAAVMWRR KSSDRKGGSY TQAASSDSAQ GSDVSLTACK V
```

```
                        -20                -15                -10                 -5                  1
A*110101  ATG GCC GTC ATG GCG CCC CGA ACC CTC CTC CTG CTA CTC TCG GGG GCC CTG GCC CTG ACC CAG ACC TGG GCG G|GC

                     5                  10                 15                 20                 25
A*110101  TCC CAC TCC ATG AGG TAT TTC TAC ACC TCC GTG TCC CGG CCC GGC CGC GGG GAG CCC CGC TTC ATC GCC GTG GGC

                    30                 35                 40                 45                 50
A*110101  TAC GTG GAC GAC ACG CAG TTC GTG CGG TTC GAC AGC GAC GCC GCG AGC CAG AGG ATG GAG CCG CGG GCG CCG TGG

                    55                 60                 65                 70                 75
A*110101  ATA GAG CAG GAG GCG CCG GAG TAT TGG GAC CAG GAG ACA CGG AAT GTG AAG GCC CAG TCA CAG ACT GAC CGA GTG

                    80                 85                 90                 95                100
A*110101  GAC CTG GGG ACC CTG CGC GGC TAC TAC AAC CAG AGC GAG GAC G|GT TCT CAC ACC ATC CAG ATA ATG TAT GGC TGC

                   105                110                115                120                125
A*110101  GAC GTG GGG CCG GAC GGG CGC TTC CTC CGC GGG TAC CGG CAG GAC GCC TAC GAC GGC AAG GAT TAC ATC GCC CTG

                   130                135                140                145                150
A*110101  AAC GAG GAC CTG CGC TCT TGG ACC GCG GCG GAC ATG GCA GCT CAG ATC ACC AAG CGC AAG TGG GAG GCG GCC CAT

                   155                160                165                170                175
A*110101  GCG GCG GAG CAG CAG ACA GCC TAC CTG GAG GGC CGG TGC GTG GAG TGG CTC CGC AGA TAC CTG GAG AAC GGG AAG

                   180                185                190                195                200
A*110101  GAG ACG CTG CAG CGC ACG G|AC CCC CCC AAG ACA CAT ATG ACC CAC CAC CCC ATC TCT GAC CAT GAG GCC ACC CTG

                   205                210                215                220                225
A*110101  AGG TGC TGG GCC CTG GGC TTC TAC CCT GCG GAG ATC ACA CTG ACC TGG CAG CGG GAT GGG GAG GAC CAG ACC CAG

                   230                235                240                245                250
A*110101  GAC ACG GAG CTC GTG GAG ACC AGG CCT GCA GGG GAT GGA ACC TTC CAG AAG TGG GCG GCT GTG GTG GTG CCT TCT

                   255                260                265                270                275
A*110101  GGA GAG GAG CAG AGA TAC ACC TGC CAT GTG CAG CAT GAG GGT CTG CCC AAG CCC CTC ACC CTG AGA TGG G|AG CTG

                   280                285                290                295                300
A*110101  TCT TCC CAG CCC ACC ATC CCC ATC GTG GGC ATC ATT GCT GGC CTG GTT CTC CTT GGA GCT GTG ATC ACT GGA GCT

                   305                310                315                320                325
A*110101  GTG GTC GCT GCC GTG ATG TCG AGG AGG AAG AGC TCA G|AT AGA AAA GGA GGG AGT TAC ACT CAG GCT GCA A|GC AGT

                   330                335                340

A*110101  GAC AGT GCC CAG GGC TCT GAT GTG TCT CTC ACA GCT TGT AAA G|TG TGA
```

Figure 21. The amino acid (upper panel) and nucleotide (lower panel) sequence of HLA A*110101. The wild-type sequence is illustrated with the region into which the mutation was introduced to produce the CD8-non-binding version highlighted in red. Sequence from the IMGT/HLA database.

```
                    -21       -11       -1        10        20        30        40        50        60        70
B*070501            MLVM APRTVLLLLS AALALTETWA GSHSMRYFYT SVSRPGRGEP RFISVGYVDD TQFVRFDSDA ASPREEPRAP WIEQEGPEYW DRNTQIYKAQ

                    80        90        100       110       120       130       140       150       160       170
B*070501            AQTDRESLRN LRGYYNQSEA GSHTLQSMYG CDVGPDGRLL RGHNQYAYDG KDYIALNEDL RSWTAADTAA QITQRKWEAA REAEQRRAYL EGECVEWLRR

                    180       190       200       210       220       230       240       250       260       270
B*070501            YLENGKDKLE RADPPKTHVT HHPISDHEAT LRCWALGFYP AEITLTWQRD GEDQTQDTEL VETRPAGDRT FQKWAAVVVP SGEEQRYTCH VQHEGLPKPL

                    280       290       300       310       320       330       340
B*070501            TLRWEPSSQS TIPIVGIVAG LAVLAVVVIG AVVAAVMCRR KSSGGKGGSY SQAACSDSAQ GSDVSLTA
```

```
                              -20               -15               -10               -5                1
B*070501            ATG CTG GTC ATG GCG CCC CGA ACC GTC CTC CTG CTG CTC TCG GCG GCC CTG GCC CTG ACC GAG ACC TGG GCC G|GC
                                5                 10                15                20                25
B*070501            TCC CAC TCC ATG AGG TAT TTC TAC ACC TCC GTG TCC CGG CCC GGC CGC GGG GAG CCC CGC TTC ATC TCA GTG GGC
                               30                35                40                45                50
B*070501            TAC GTG GAC GAC ACC CAG TTC GTG AGG TTC GAC AGC GAC GCC GCG AGT CCG AGA GAG GAG CCG CGG GCG CCG TGG
                               55                60                65                70                75
B*070501            ATA GAG CAG GAG GGG CCG GAG TAT TGG CAC CGG AAC ACA CAG ATC TAC AAG GCC CAG GCA CAG ACT GAC CGA GAG
                               80                85                90                95               100
B*070501            AGC CTG CGG AAC CTC CGC GGC TAC TAC AAC CAG AGC GAG GCC G|GG TCT CAC ACC CTC CAG AGC ATG TAC GGC TGC
                              105               110               115               120               125
B*070501            GAC GTG GGG CCG GAC GGG CGC CTC CTC CGC GGG CAT AAC CAG TAC GCC TAC GAC GGC AAG GAT TAC ATC GCC CTG
                              130               135               140               145               150
B*070501            AAC GAG GAC CTG CGC TCC TGG ACC GCC GCG GAC ACG GCG GCT CAG ATC ACC CAG CGC AAG TGG GAG GCG GCC CGT
                              155               160               165               170               175
B*070501            GAG GCG GAG CAG CGG AGA GCC TAC CTG GAG GGC GAG TGC GTG GAG TGG CTC CGC AGA TAC CTG GAG AAC GGG AAG
                              180               185               190               195               200
B*070501            GAC AAG CTG GAG CGC GCT G|AC CCC CCA AAG ACA CAC GTG ACC CAC CAC CCC ATC TCT GAC CAT GAG GCC ACC CTG
                              205               210               215               220               225
B*070501            AGG TGC TGG GCC CTG GGT TTC TAC CCT GCG GAG ATC ACA CTG ACC TGG CAG CGG GAT GGC GAG GAC CAA ACT CAG
                              230               235               240               245               250
B*070501            GAC ACT GAG CTT GTG GAG ACC AGA CCA GCA GGA GAT AGA ACC TTC CAG AAG TGG GCA GCT GTG GTG GTG CCT TCT
                              255               260               265               270               275
B*070501            GGA GAA GAG CAG AGA TAC ACA TGC CAT GTA CAG CAT GAG GGG CTG CCG AAG CCC CTC ACC CTG AGA TGG G|AG CCG
                              280               285               290               295               300
B*070501            TCT TCC CAG TCC ACC ATC CCC ATC GTG GGC ATT GTT GCT GGC CTG GCT GTC CTA GCA GTT GTG GTC ATC GGA GCT
                              305               310               315               320               325
B*070501            GTG GTC GCT GCT GTG ATG TGT AGG AGG AAG AGT TCA G|GT GGA AAA GGA GGG AGC TAC TCT CAG GCT GCG T|GC AGC
                              330               335               340
B*070501            GAC AGT GCC CAG GGC TCT GAT GTG TCT CTC ACA GCT TGA
```

Figure 22. The amino acid (upper panel) and nucleotide (lower panel) sequence of HLA B*070501. The wild-type sequence is illustrated with the region into which the mutation was introduced to produce the CD8-non-binding version highlighted in red. Sequence from the IMGT/HLA database.

pGMT7 plasmids containing the original templates were used to transform DH5α *E.coli*. The plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Germany) and sequenced. Primers containing the desired mutation were designed (Figure 23), produced commercially by Invitrogen, UK and used in the QuikChange II kit (Stratagene, California, USA) according to the manufacturer's instructions. Briefly, the following were prepared on ice: 125ng of primer sense (S), 125ng of

primer antisense (AS), 5µl of proprietary buffer, 1µl of deoxyribonucleotide triphosphate

A) B*0702 to B*0705

Original sequence	GGG CGC CTC CTC CGC GGG CAT GAC CAG TAC GCC TAC GAC GGC
Primer S	5′ -C CTC CGC GGG CAT AC CAG TAC GCC TAC- 3′
Primer AS	5′ -GTA GGC GTA CTG GT ATG CCC GCG GAG G- 3′

B) B*0705 to B*0705 CD8 non-binding

Original sequence	GGC GAG GAC CAA ACT CAG GAC ACT GAG CTT GTG GAG ACC AGA
Primer S	5′ -GAG GAC CAA ACT CAG GAG CTT GTG GAG ACC- 3′
Primer AS	5′ -GGT CTC CAC AAG CTC CTG AGT TTG GTC CTC- 3′

C) A*1101 to A*1101 CD8 non-binding

Original sequence	GGG GAG GAC CAG ACC CAG GAC ACG GAG CTC GTG GAG ACC AGG
Primer S	5′ -GAG GAC CAG ACC CAG GAG CTC GTG GAG ACC AGG- 3′
Primer AS	5′ -CCT GGT CTC CAC GAG CTC CTG GGT CTG GTC CTC- 3′

Figure 23. The portion of the original sequence and the primers required to introduce the necessary changes using site directed mutagenesis. A) Producing B*0705 from 0702 requires a changes from aspartic acid (D) to asparagine (N). D is encoded by GAC, N by AAC. B) Producing B*0705 CD8 non-binding molecules requires changing DT (GACACT) to KA (AAAGCC). C) Producing A*1101 CD8 non-binding molecules requires changing DT (GACACG in this case) to KA (AAAGCC).

(dNTP) mix, dsDNA template in different concentrations from 10 to 100ng, 1µl Pfu Ultra polymerase and ddH_20 to a volume of 40µl. Tubes were then run in the following polymerase chain reaction programme:

1. 95°C	30 seconds	
2. 95°C	30 seconds	
3. 55°C	1 minute	
4. 68°C	4 minutes	Back to step 2 - 12 times.

Control tubes were also run using a proprietary pWhitescript plasmid. 1µl of Dpn1 enzyme was added to each tube, mixed well, and then left at 37°C for 1 hour to digest parental DNA. The mutated plasmids were then used to transform XL-Blue cells (Stratagene) using a heat-shock technique as per the manufacturers instructions. Transformed cells were inoculated onto LB agar plates containing ampicillin, isopropyl thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and incubated at 37°C overnight. 10-20 colonies were then selected from successful plates and used to inoculate both a 15ml tube containing 4ml LB/ampicillin culture media and a PCR tube containing 20µl of water. To the PCR tube was added 3µl of proprietary buffer, 3µl of 2.5mM dNTP, 0.6µl 0.1M DTT, 3µl of 5µM pGMT7 sense primer (T7: TAATACGACTCACTATAGGG), 3µL of 5µM pGMT7 antisense primer (OX281: AGCAAAAAACCCCTCAAGACCCG), 7.3µl of ddH_20 and 0.1µl of SuperTaq polymerase (HT Biotechnology, Cambridge, UK). They were run using the following PCR programme:

1. 94°C	10 minutes	
2. 94°C	30 seconds	
3. 54°C	30 seconds	
4. 72°C	75 seconds	Back to step 2 – 35 times.
5. 72 °C	10 minutes	
6 4°C	Hold	

A fraction of each reaction was then mixed with gel loading buffer (Eurogentec, Belgium) and run on a 0.8% agarose electrophoresis gel looking for the 800 base pair product. Those samples with an 800 base pair product were grown in the

LB/ampicillin broth overnight and the plasmids extracted by Miniprep for sequencing. The sequences were reviewed and the colony with the highest quality sequence and containing the desired mutation selected as the expression template. This plasmid was used to transform BL21 cells (Stratagene, California, USA) using a heat shock technique. These cells were in turn inoculated onto LB/Ampicillin agar plates and a colony selected the following day to grow for protein expression and refolding as described below.

Expression of monomers and β2-microglobulin

BL21 cells containing the plasmid with either the heavy chain or β2-microglobulin sequence were grown overnight in 250mls of LB-ampicillin at 37°C. 50ml of this was inoculated into 1l of low salt LB/ampicillin culture media the following morning and incubated with shaking at 37°C. The optical density as compared to sterile LB media was checked regularly. Once an OD of over 0.6 was reached (indicative of the phase of exponential growth – took around 3 hours) 0.5ml of 1M IPTG was added to induce protein expression. 6 hours later the cells were spun down at 4°C and washed in sterile phosphate buffered saline (PBS). This suspension was sonicated (MISONIX XL2020 Sonicator with titanium 0.5 inch horn) in a glass beaker on ice to prevent sample heating until the solids were lysed and the mix ran like water. This was centrifuged at 15000 rpm for 20 minutes in a precooled 4°C centrifuge. Supernatant was discarded and solids washed in a Triton-based wash, resuspended in a glass homogeniser and then spun once again at 15000 rpm for 20 minutes. This was done 3 times. After the final wash the solid material was resuspended in Triton-free wash solution. This was then spun at 15000 rpm for 10 minutes and the solid material resuspended in a urea

based protein-solubilisation mix. This was left rolling overnight at 4°C to allow the protein to dissolve. Insoluble remnants were removed by centrifugation and the monomer protein-containing supernatant frozen at -80°C. Protein concentration was assessed using the Bio-Rad colormetric protein assay according to the manufacturer's instructions.

Refolding the heavy chain, β2-microglobulin and peptide and isolating the monomer

10mg of β2-microglobulin was thawed, placed in a universal container and 6 ml of refold buffer (see chapter 2) added slowly at 4°C whilst stirring constantly to avoid precipitation. This was added directly to 500ml of refold buffer at 4°C. 10ml of refold buffer was added slowly with shaking to a universal tube containing 30mg of heavy chain and 10mg of the appropriate peptide epitope (resuspended in around 200µl DMSO). This mixture was then added to the β2-microglobulin/refold buffer mix. This was left stirring at 4°C for 48 hours. Refolded monomeric complexes were then concentrated using a nitrogen pressured Amicon stir cell concentrator with a 10000 MW filter (Millipore, Massachusetts, USA) to achieve a final volume of around 5ml. Buffer exchange was then performed using PD10 columns (Amersham Biosciences) and BirA buffer (1ml 2M Tris, 0.5ml 5M NaCal, 187.5µl 4M magnesium chloride, made up to 100ml with H_2O). Buffer exchange produced a final volume of 7mls and this was biotinylated overnight through the addition of Bir A enzyme (Avidity - 875µl Biomix A, 875µl Biomix B, 5µl Bir A enzyme, 100µl dBiotin) and the resulting biotinylated monomer purified by FPLC (Amersham biosciences) using a G75 superdex column. The refolded complex eluted at around 150ml (see example trace in

Figure 24). The fraction containing the refolded monomer was concentrated to a volume of 1ml using a 10000 MW centrifugal concentrator (Millipore) and the final protein concentration measured using a colormetric based assay (Bio-Rad laboratories). 0.5µl of 0.5M EDTA and 1µl of each of two protease inhibitors, leupeptin and pepstatin (Sigma), were added to each 1ml of monomer before dividing into 50µg aliquots and freezing at -80°C.

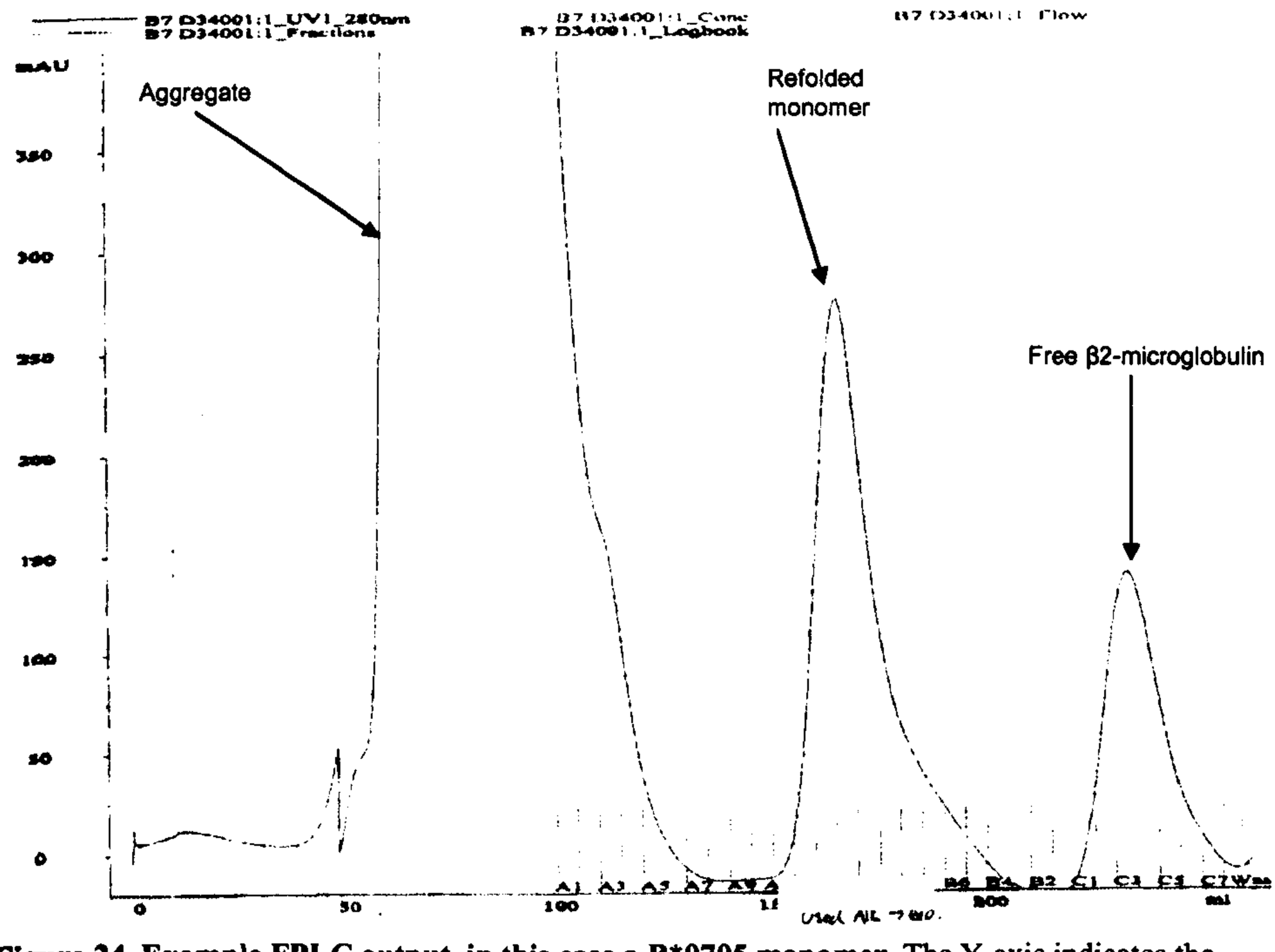


Figure 24. Example FPLC output, in this case a B*0705 monomer. The Y-axis indicates the presence of protein as detected by UV laser. The X-axis indicates the volume of FPLC buffer that has passed through the column in mls.

Labelling tetramers

A new tetramer required titration with potential fluorochromes. The concentration of flurochrome required to bind all monomer but not have unbound conjugate was determined through a modified enhanced chemiluminescence (ECL) assay. Briefly,

50µg of tetramer was thawed and mixed with titrations of fluorochrome and rolled in the dark at 4°C for 1 hour. 1µl of each would then be placed on Hybond protein binding paper (Amersham biosciences, USA) together with 1µl of monomer, 1µl of biotin (Mabtech, Sweden) as a positive control, and 1µl of water as a negative control. Once dry the paper was covered with 4% powdered milk solution for 15 minutes with shaking to occupy remaining protein binding sites. The paper was washed 3 times with PBS/0.05% Tween and then covered with 1:15000 horseradish peroxidise (BD biosciences, USA) in PBS for 15 minutes with shaking. The paper was washed 3 times with PBS/0.05% Tween and then covered with a 50:50 mix of ECL reagents A and B (Amersham biosciences). The paper was drained after 1 minute and then exposed to X-ray film in a dark room. Spots giving a signal still had unoccupied biotin and were not sufficiently saturated. The minimum concentration of fluorochrome causing loss of signal was used for further conjugations.

Thereafter when required an aliquot was thawed and combined with the relevant fluorochrome – either phycoerythrin (PE)-labelled, Quantum red-labelled (Sigma) or allophycocyanin (APC)-labelled streptavidin (Molecular Probes) at a 4:1 molar ratio to form tetramers. To achieve maximal saturation the fluorochrome would be added to the monomer in 4 portions every 30 minutes and then left rolling in the dark overnight at 4°C. Conjugated tetramers were kept at 4°C. The tetramers were conjugated with different fluorochromes to allow co-staining of T cell populations.

Testing the tetramers

The use of the CD8-non-binding tetramers is described below. The B*0705 tetramer was refolded with peptides representing a previously described epitope(187) that is the same for DEN-2, 3, and 4 (APTRVVAAEM) but differs for DEN-1 (APTRVVASEM). The monomer was conjugated with APC and the tetramer used to stain 5 B0705 positive patients together with CD38 FITC and CD8 PE-Cy7 as described above. 2 samples were taken within the first week of illness and 3 from between 14 and 21 days (Figure 25).

PT ID	Infecting serotype	Illness severity	Days since admission
BC411	unknown	DHF II	4
DF163	unknown	DHF III	3
BC385	D2	DHF II	14
BC389	D2	DHF II	14
BC403	D4	DHF II	21

Figure 25. B*0705 positive patient's clinical data.

No patient demonstrated any DEN-1 positive populations (data not shown). However all patients had small DEN-2/3/4 tetramer-positive populations. Tetramer-positive populations from samples taken within the first week of illness had higher levels of CD38 expression (Figure 26). This increased level of activation has been observed in patients recognising other dengue tetramers and is discussed further below.

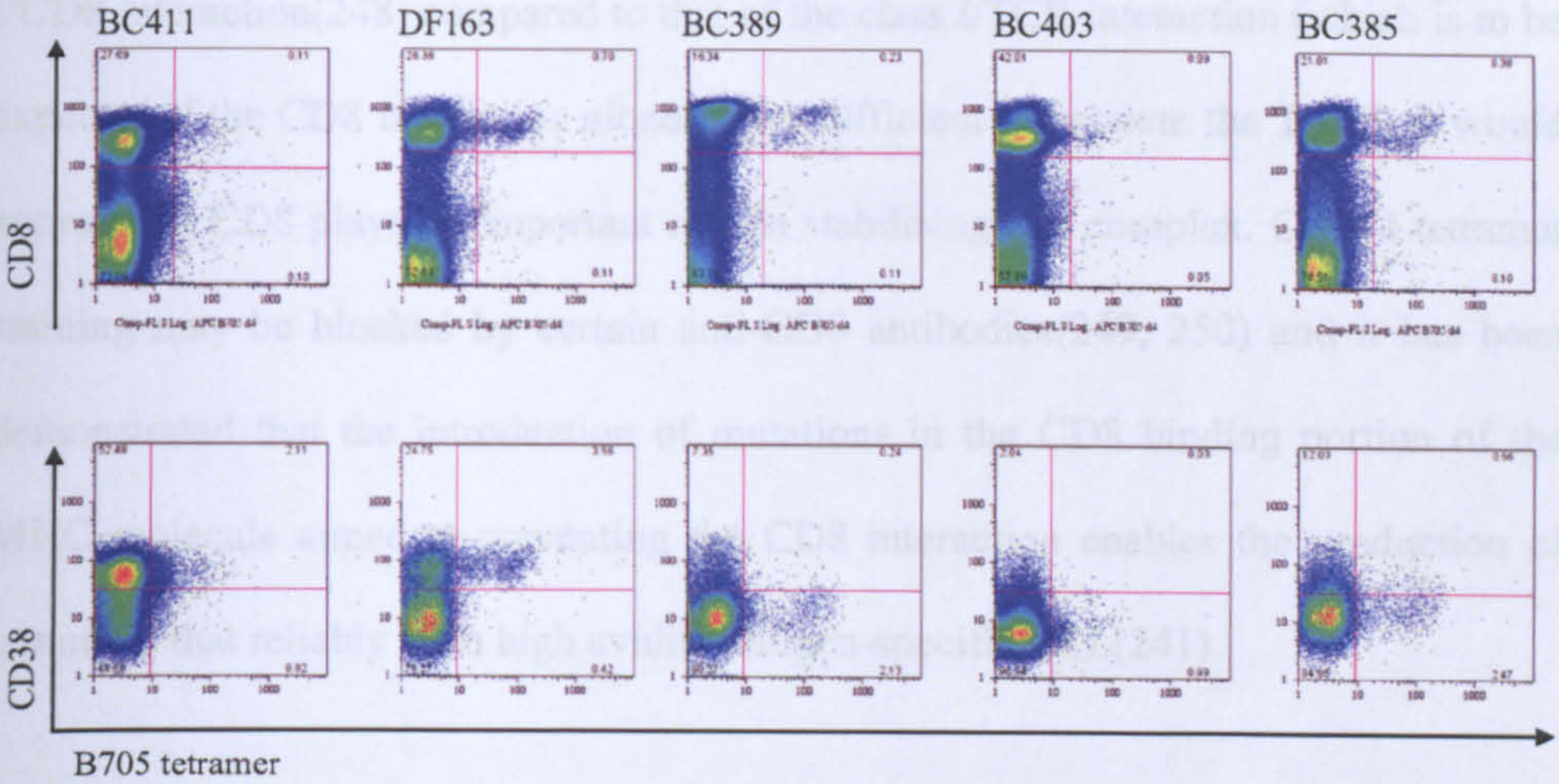


Figure 26. PBMC from B*0705 positive patients stained with B*0705 tetramers and CD38 FITC and CD8 PE-Cy7. These plots are gated on lymphocytes. BC411 and DF163 samples were taken within the first 5 days of admission the remainder are from samples taken 2-3 weeks after admission.

Discussion

Several factors influence the avidity of the TCR/peptide-MHC class I interaction. These include the respective density of TCR on the target cell and peptide-MHC on the lymphocytes, the stability of the complex that is formed between them(243), and the presence of CD8 together with the TCR in the immune synapse(244). Generally the intensity of cell staining by tetramers has been considered a "surrogate marker" of TCR affinity(245). This however may be influenced by other factors such as the density of TCR expression on the cell surface and tetramer internalisation(246).

As described above the process of antigen recognition involves engagement of both TCR and either the CD8 or CD4 co-receptor to the peptide-MHC complex. CD4 molecules do not appear to enhance the stability of this interaction(247) and appear to be more involved with the intracellular activation pathway than they do stabilisation of the extracellular trigger. In contrast despite the relatively low affinity of the class

I/CD8 interaction(248) compared to that of the class I/TCR interaction (which is to be expected if the CD8 interaction alone is not sufficient to activate the T cell) it would appear that CD8 plays an important role in stabilising this complex. Class I tetramer staining may be blocked by certain anti-CD8 antibodies(249, 250) and it has been demonstrated that the introduction of mutations in the CD8 binding portion of the MHC molecule aimed at preventing the CD8 interaction enables the production of tetramers that reliably stain high avidity antigen-specific CTL(241).

The mutation(241) introduced into the tetramers described in this chapter allows the detection and quantification of antigen-specific CTL of sufficiently high TCR/peptide-MHC avidity that they do not require the additional component of the CD8 interaction. Such high avidity cells have been shown to be important in the clearance of viral infection but their affinity for antigen appears to render them more vulnerable to activation induced cell death(251, 252). It is possible that just as such high affinity CTLs are important in viral clearance they may play a similarly significant part in the generation of immunopathology (discussed in detail above). The CD8-non-binding tetramers described here will allow the high avidity fraction of antigen-specific CTL populations to be tracked throughout disease to convalescence and perhaps even allow such observations to be related to disease severity.

CHAPTER 4 HIGH AVIDITY CROSS-REACTIVE CD8+ CYTOTOXIC T-CELLS IN THE PATHOGENESIS OF ACUTE SECONDARY DENGUE VIRUS INFECTION

Introduction

Scott Halstead first demonstrated that the vast majority of cases of severe dengue disease were occurring in those patients experiencing secondary infection – individuals remained immune to the viral serotype causing their first infection but were at risk of severe disease on infection with heterologous DEN serotypes (1, 7). As discussed above antibody dependent enhancement has been widely accepted as a good explanation of the link between immunological cross-reactivity and disease pathogenesis and there is evidence to support this hypothesis. However additional mechanisms are likely to be involved to account for the complex clinical phenotype of dengue disease. Cross-reactive cellular immune responses to dengue virus have been linked with pathogenesis (189, 222, 253) and there is great complexity in the response of serotype cross-reactive memory CTL to heterologous variant peptides. It is likely that cross-reactive memory T cells might demonstrate distinctive phenotypic features (e.g. altered cytokine profiles) that could contribute to induction of plasma leakage (222-224). Tetramers provide a powerful means of assessing the cross-reactivity and avidity of CTL populations from dengue patients and relate this to their phenotype. This chapter concerns work that sought to test the hypothesis that cross-reactive CTLs derived from memory populations generated by primary infection had a distinctive phenotype and might contribute to the pathology of severe secondary infection.

Previous work

Dr Tao Dong, working in the Oxford MRC Human Immunology Unit and the Oxford University Clinical Research Unit, Ho Chi Minh City, Viet Nam, studied the CD8+ T cell responses to a previously identified immunodominant NS3 epitope (Figure 27, and referred to below as the "GTS epitope") presented by HLA-A*11 (189) – the most common HLA allele in Viet Nam. This epitope was chosen as it varied between three of the four serotypes. Tetramers assembled using peptides representing it could be used to assess the presence and extent of serotype-cross-reactivity demonstrated by CTLs from dengue patients (termed the "GTS tetramers" below). Her work provided the foundations for the material that follows in this chapter and is summarised briefly here (p.118-126).

Peptide	Serotype	NS3 amino acid sequence
pD1	Dengue 1	**GTSGSPIVNR**
pD2	Dengue 2	**GTSGSPIIDK**
pD3/4	Dengue 3 and 4	**GTSGSPIINR**

Figure 27. An immunodominant HLA-A11 epitope from the dengue virus NS3 protein.

Patients recruited

20 HLA-A*1101 positive dengue patients were screened for responses to the A*11 epitope given above. 6 individuals with confirmed infecting dengue serotypes, epitope-specific responses detected either acutely or in convalescence and known to be experiencing secondary infection (Figure 28) were recruited for these studies. 3 of these (BC307, MD856 and MD893) had specific responses in both acute and convalescent samples.

PT ID	Age	Infecting serotype	Illness severity	Days of illness
BC307	17	unknown	DHF	6
MD856	11	D4	DF	5
MD893	14	D4	DF	3
MD907	19	D3	DF	3
MD899	9	D4	DF	4
MD881	14	D1	DF	2

Figure 28. Clinical data of those patients involved in this study.

Limitations of ex vivo tetramer staining

Ex vivo PBMC samples were double-stained with tetramers assembled with different epitopes and labelled with different fluorochromes to allow assessment of CTL cross-reactivity. The dengue 2 epitope varies between viral strains. The **GTSGSPIIDK** variant was used in these studies. Great variation was found in the fraction of tetramer positive cells within acute samples analysed *ex vivo*. Patients presenting to hospital early (mostly children) had lower frequencies than those presenting later (usually adults). One adult patient (BC307) demonstrated large ex vivo tetramer positive populations acutely (Figure 29 – panel A) when approximately 20% of tetramer positive cells were cross-reactive, recognising both the pD2 and pD3/4 tetramers. This population was not detectable in the convalescent (day 21) sample. The majority of patients however showed much less significant staining, a phenomenon that may be related to acute down-regulation of cell-surface TCR expression [4].

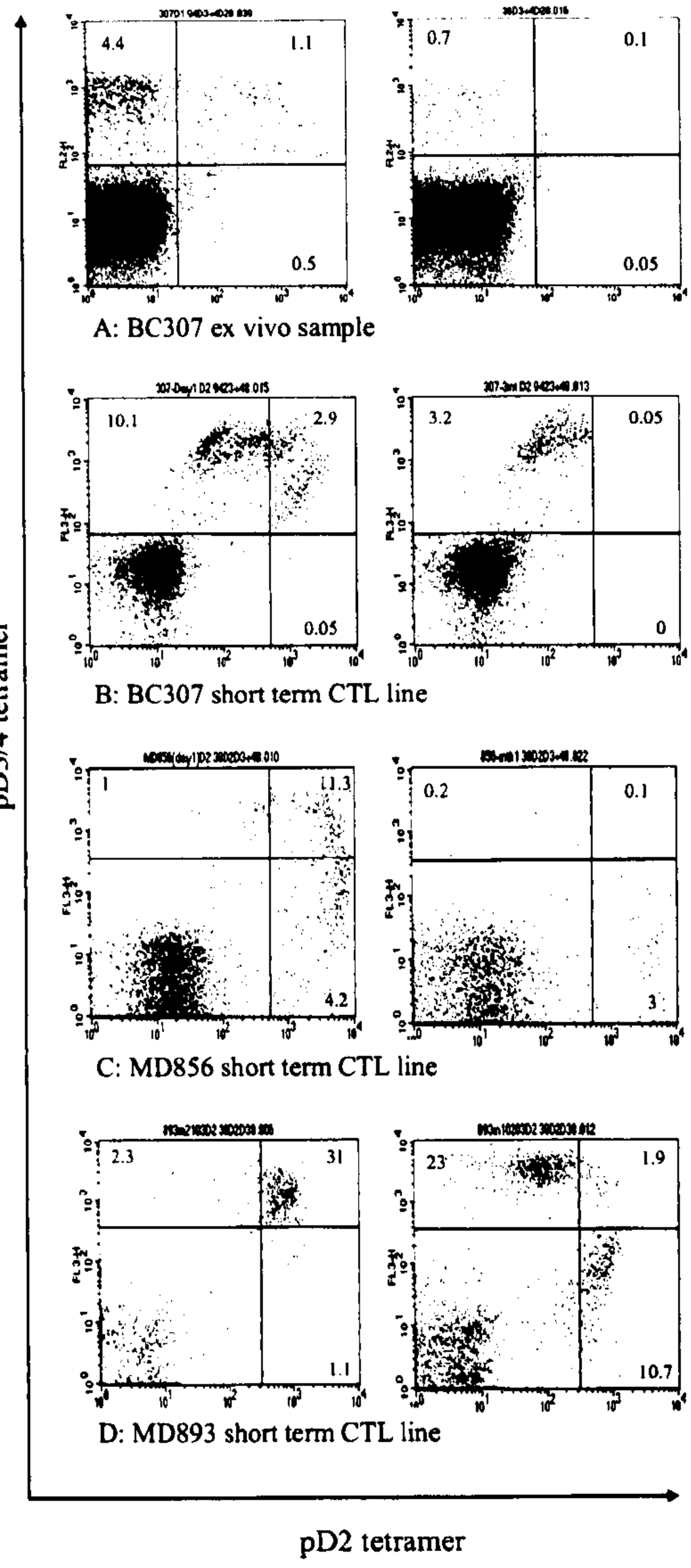


Figure 29. A) Ex vivo pD2 and pD3/4 tetramer staining of PBMC from patient BC307. PBMCs from BC307 were stained with A*11 pD3/4 and pD2 tetramers and CD8 antibody. Plots are gated on CD8 positive lymphocytes. Most cells in the acute sample (left) are specific for pD3/4 but there is a significant population cross-reactive with this and pD2. Only pD3/4 specific cells are detected in convalescence (right). B,C,D) Highly cross-reactive T cells can be expanded from acute but not convalescent PBMC from dengue patients. Short term CTL lines were generated by pulsing PBMC with 2μM of pD2 and stained with pD2 and pD3/4 tetramers on either the 14th (BC307) or 20th (MD856, MD893) day after stimulation. The highly cross-reactive populations apparent in the acute sample are not detectable in the convalescent sample taken one month later when more serotype specific populations have appeared. Data courtesy of Dr Tao Dong.

Generation of short-term CTL lines results in expansion of epitope-specific cell populations

To assess the fine specificities of dengue-specific CTL with limited cell numbers, and in the face of this likely TCR down-regulation short-term CTL lines were grown by stimulating PBMCs with each of the A*11 NS3 epitope variants. Such stimulations produced large expansions in tetramer positive populations. On day 20 after stimulation CTL were double-stained as above. At all time-points stimulation with the pD3/4 peptide resulted in the expansion of cells mostly specific for pD3/4 (data not shown). However stimulation with pD2 resulted in expansions of CTL binding both D2 and D3/4 tetramers equally well from PBMCs of four of the patients with acute DEN3 or DEN4 infection (MD893, MD856, BC307, MD881).

Highly cross-reactive CTL can be expanded from acute, but not convalescent samples.

The pattern of staining differed slightly in each patient but all 4 demonstrated expansions of highly cross-reactive cells from acute samples. CTL recognising both pD2 and pD3/4 were considered to be highly cross-reactive given their equal recognition of both tetramers despite significant sequence variation. CTL recognising pD1 usually recognised pD3/4 – a reflection of their greater homology. Cells showing high cross reactivity between pD2 and pD3/4 always cross reacted with pD1. Consistent with the *ex vivo* BC307 observation above, these highly cross-reactive CTL were undetectable in, and could not be expanded from the convalescent samples of patient MD893, MD856 and BC307 (Figure 29 – panels B, C and D) - no convalescent sample was available for MD881. Only CTL showing specificity for a

single serotype or limited cross-reactivity (i.e. strong binding of the tetramer folded with the peptide of one serotype, weak binding of the other) remained. There were 2 patients in whom this phenomenon was not observed (data not shown): antigen specific cells could not be detected acutely in patient MD899 but partially cross-reactive populations recognising pD2 and pD3/4 were apparent by convalescence; patient MD907 had only serotype specific cells present acutely (pD2).

Highly cross-reactive CTL express higher levels of CD38.

Ex vivo staining of PBMC from patient BC307 demonstrated equally high levels of CD38 staining among all tetramer positive cells in the acute phase (Figure 30, panel A). By three weeks after the acute sample CD38 expression had fallen markedly. At this time point CD8+ cells recognising the two similar peptide variants, pD1 and pD3/4, were present (partially cross-reactive – figure 30, panel B) but cells cross-reactive between the more diverse peptides, pD2 and pD3/4 could not be detected (highly cross-reactive cells – Figure 29 panel A). CD38 expression was greater on those T cells recognising both pD1 and pD3/4 (57% CD38 high – figure 30 panel D) than upon partially or non cross-reactive T cells (19% CD38 high – figure 30 panel C). This suggests that highly cross-reactive T cell populations are more activated than cells exhibiting low levels of cross-reactivity. It is not clear why pD1-pD3/4 cross-reactive cells are present at 3 weeks and pD2-pD3/4 cross-reactive cells are not. It could be speculated that this highly cross-reactive latter group experiences even higher levels of activation resulting in activation induced cell death and clonal deletion.

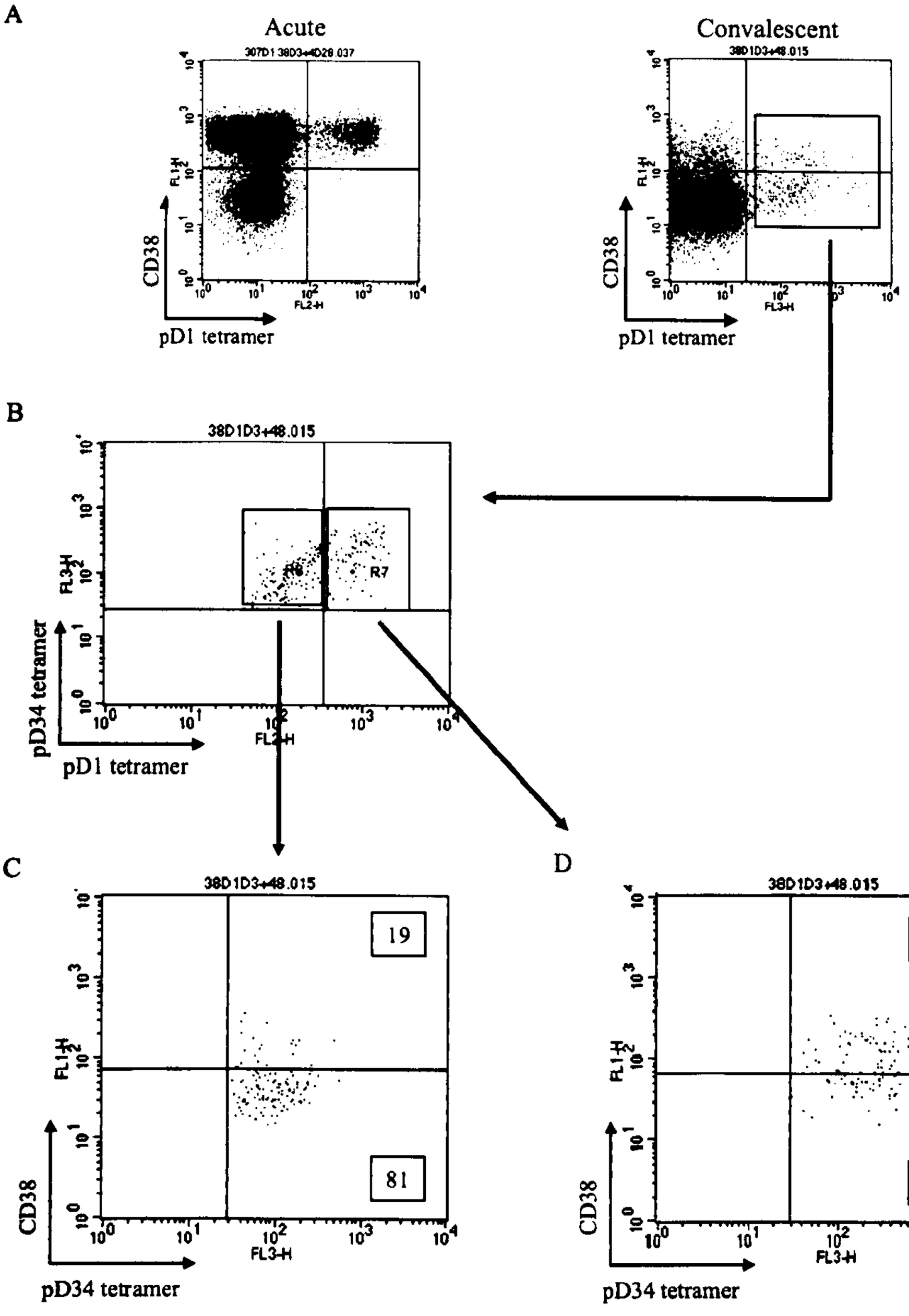


Figure 30. Highly cross-reactive CTL are more activated than partially cross-reactive CTL Frozen PBMC from patient BC307 on day 21 of illness were stained with pD1 and pD3/4 tetramers together with CD38 and CD8. (A) CD38 staining of the whole CD8+ population acutely and at day 21. **(B)** Lymphocytes gated on CD8+ tetramer+ cells and co-stained with pD1 and pD3/4 tetramers. Highly cross-reactive cells (R7) show higher levels of expression of CD38 (D) than partially cross-reactive cells (C). Data courtesy of Tao Dong.

Cross-reactive clones produce greater levels of inflammatory cytokines and kill more effectively at low level stimulations than serotype-specific clones.

A number of CTL clones were generated from patient BC307 of which 4 were chosen for more detailed study. Cross-reactive clones were derived from the acute sample – only serotype-specific clones or clones showing low-level cross-reactivity could be derived from the convalescent sample. Highly cross-reactive clone E5 maintained high levels of cytolytic activity at low peptide concentrations with all three peptide variants in a standard chromium release assay (Figure 31). Partially cross-reactive clone C48 showed good lytic activity against cells pulsed with pD3/4 and pD1 but low activity against pD2. Clone C42 showed good activity against pD3/4, low activity against pD1 and no recognition of pD2. Dengue serotype 3 specific clone D9 showed intermediate activity against pD3/4 and failed to recognise target cells pulsed with the peptide variants. All cross-reactive or partially cross-reactive clones maintained lytic activity against B-cells pulsed with peptide concentrations as low as 0.01μM. Serotype specific clone D9 showed no or negligible activity at these levels. Clones were stimulated with B cells pulsed with the relevant peptide and the concentration of cytokines in the tissue culture supernatant measured at 24 hours. Highly cross-reactive clone E5 consistently produced higher levels of TNF-α, IFN-γ and GM-CSF than most serotype specific clones (Figure 32) and only E5 produced IL-10.

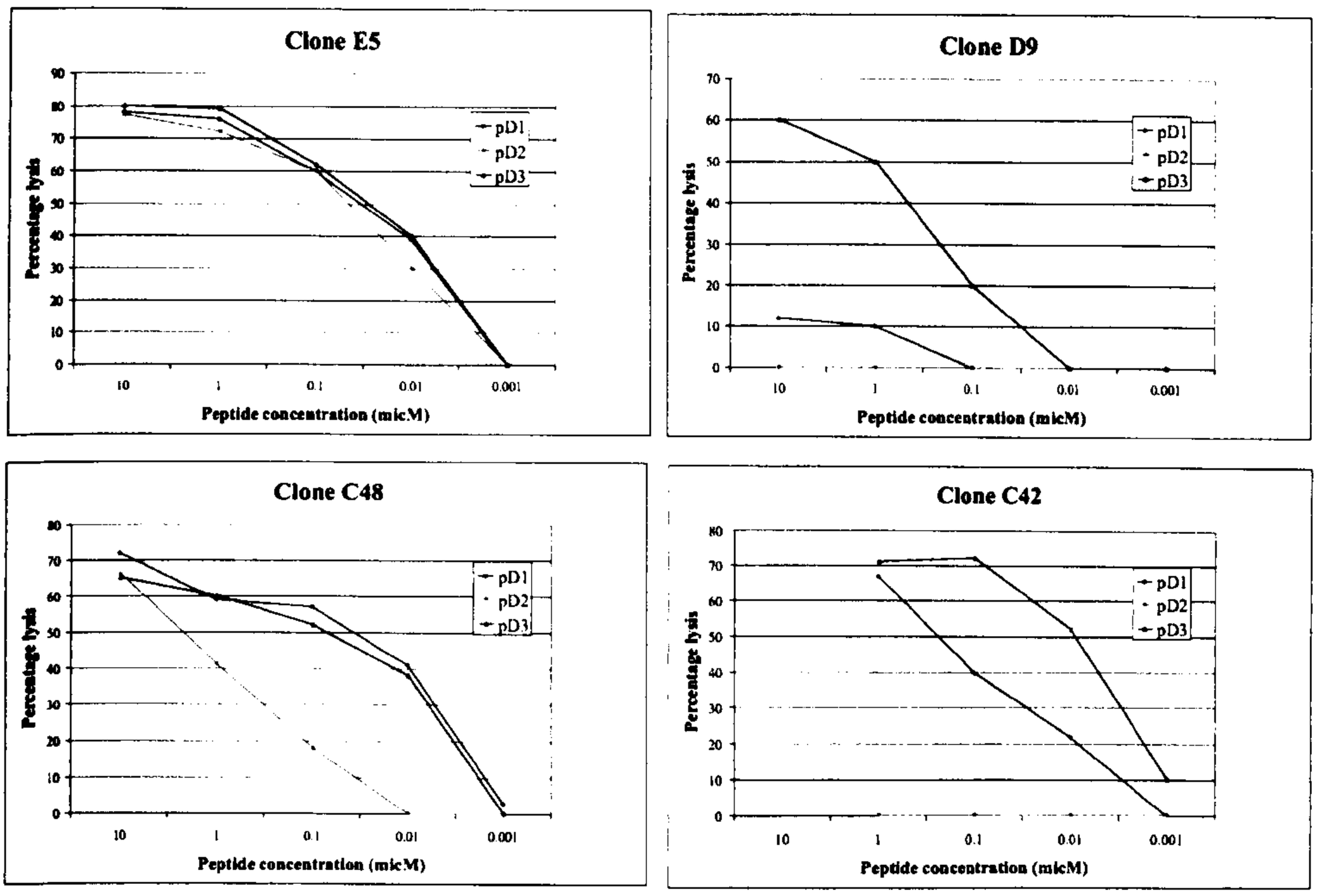


Figure 31. Clones differ in specificity and cytolytic efficacy in chromium-release assays. Cross-reactive clone E5 and partially cross-reactive clones C42 and C48 maintain their cytolytic activity at peptide concentrations as low as 0.01μM. Serotype specific clones D9 (recognising pD3/4) showed no activity at this level of stimulation. Data couresy of Dr Nguyen Vinh Chau.

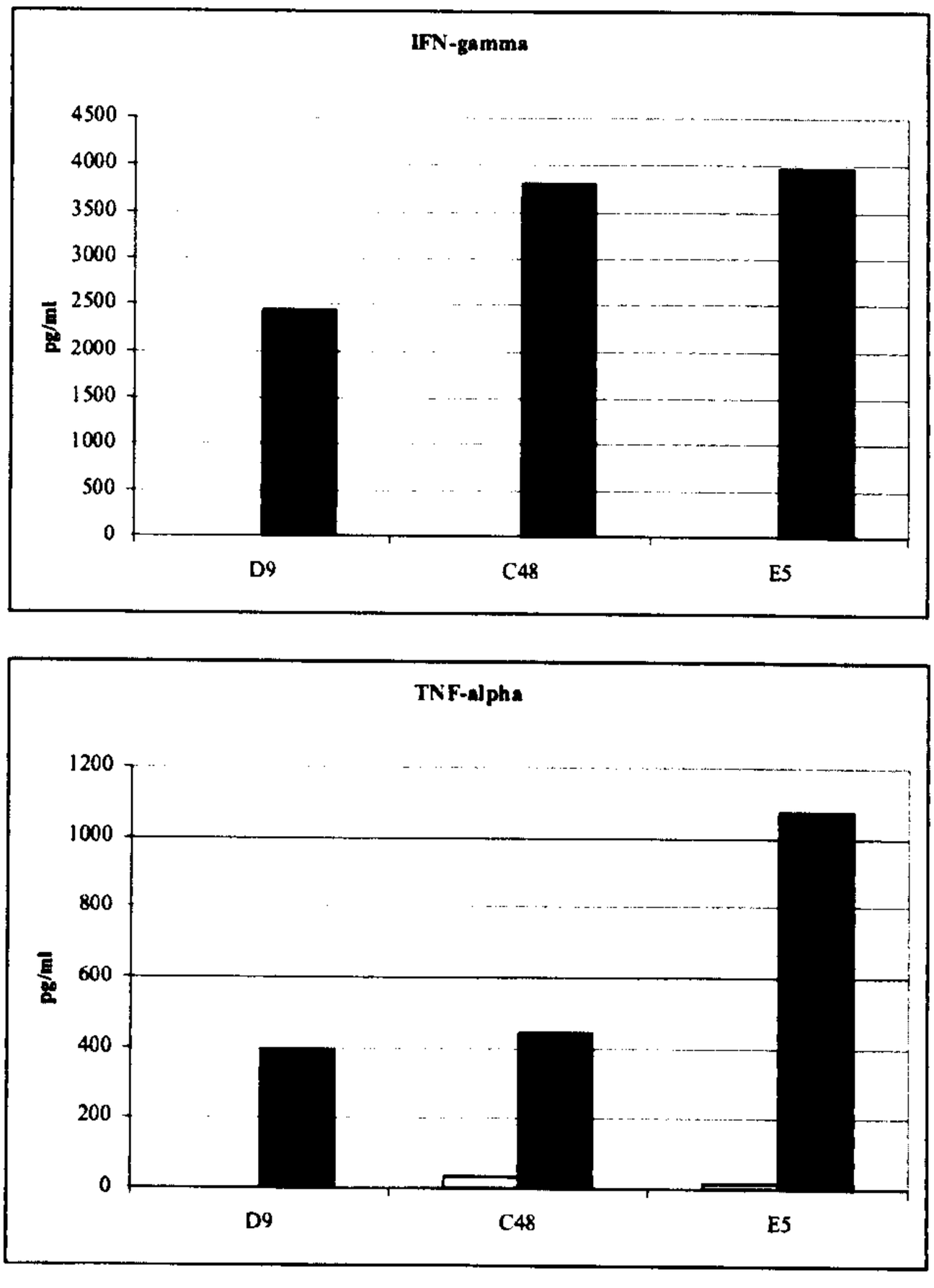


Figure 32. Cross-reactive clones produce higher levels of both type 1 and type 2 cytokines than serotype-specific clones. Cytokines produced by cross-reactive (E5), partially cross-reactive (C48) and DEN3-specific (D9) clones derived from patient BC307 stimulated with B cells pulsed with 1μM pD3/4 at an E:T ratio of 10:1. Black bar: cytokine pulsed B cells, white bar: RPMI control pulsed B cells. Data is representative of three independent experiments.

Materials and methods

Patient samples

Samples were collected in Viet Nam. Initial tetramer staining and growth of short-term lines was performed on fresh samples. The remainder of each sample was frozen and shipped to Oxford.

Generation of clones

Short-term lines were grown in Viet Nam, shipped to Oxford at 20°C where they were returned to a 37°C incubator. A portion of the culture was tetramer stained and if a specific population was present on FACS analysis enriched using MACS CD8 magnetic beads, cloned by limiting dilution and maintained by periodic restimulation as described above.

Results

Characterisation and specificity of clones generated from patient MD1413

Of several patients assessed, MD1413 demonstrated the best acute *ex vivo* tetramer staining (Figure 33). This was a 10 year old child experiencing a mild to moderate case of secondary dengue infection – serotype 2. Clones were generated as described above from a short-term line cultured for 20 days and screened at 3 weeks with the A*11 wild-type GTS tetramers. Antigen specific clones were expanded and the fine specificity of these dengue-specific clones assessed in a standard CTL lysis assay

(Figure 34). The majority of clones showed some degree of cross-reactivity with clone 9F5 the most broadly cross-reactive. Two clones showed high specificity for dengue 2: 3H9 and 10A4. These clones, together with 10H5 and 10B3, were selected for more detailed study. A table summarising the specificites of all the clones referred to in this chapter is given in Figure 35.

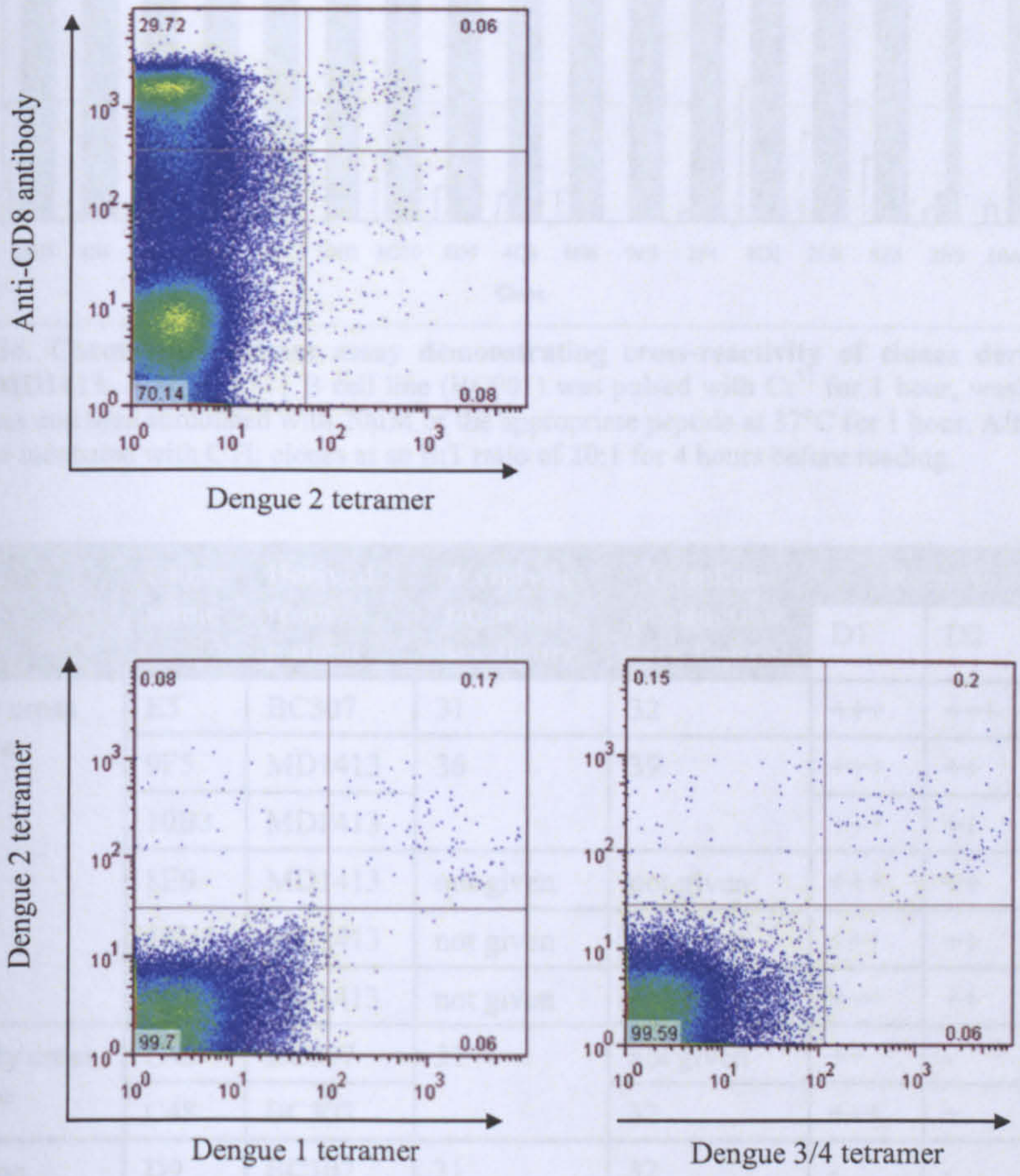


Figure 33. Ex-vivo staining of PBMC from patient MD1413 taken on day 4 of illness. PBMC gated on lymphocytes and a) stained anti-CD8 antibody and dengue 2 tetramers and b) gated on CD8 positive cells and stained with tetramer pairs. Note high proportion of cross-reactive cells together with a dengue 2 specific population.

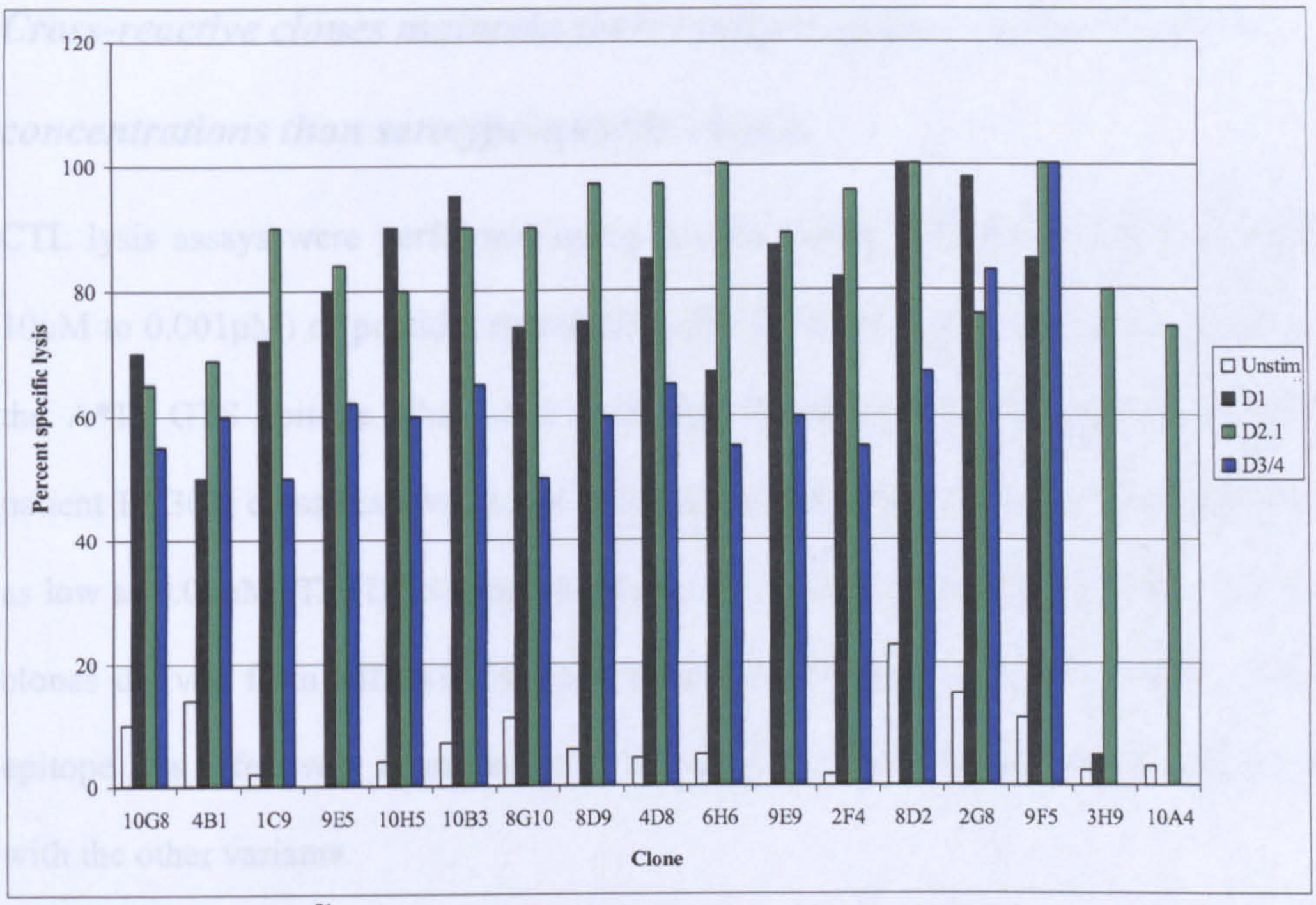


Figure 34. Chromium[51] release assay demonstrating cross-reactivity of clones derived from patient MD1413. A HLA A*11 B cell line (BC001) was pulsed with Cr^{51} for 1 hour, washed in R10 three times and then stimulated with 20μM of the appropriate peptide at 37°C for 1 hour. After washing they were incubated with CTL clones at an E:T ratio of 20:1 for 4 hours before reading.

Group	Clone name	Derived from patient	Cytolytic titrations, see figure	Cytokine production, see figure	Recognition		
					D1	D2	D3
Highly cross reactive	E5	BC307	31	32	+++	+++	+++
	9F5	MD1413	36	39	+++	++	++
	10B3	MD1413			+++	++	+++
	8E9	MD1413	not given	not given	+++	++	+++
	10H5	MD1413	not given	not given	+++	++	+++
	9E5	MD1413	not given	not given	+++	++	+++
Partially cross reactive	C42	BC307	31	not given	++	-	+++
	C48	BC307		32	+++	+	+++
Serotype specific	D9	BC307	31	32	-	-	+++
	10A4	MD1413	36	39	-	+	-
	3H9	MD1413			-	++	-

Figure 35. Summary of clones giving the patient from whom they were generated and the variants of the dengue GTS epitope recognised by each. See indicated figures for cytokine production and cytolytic titration data. Peptide recognition is classified by the percentage of specific lysis of B cells loaded with 0.1μM of peptide in a standard chromium release assay. +++ 50% or greater lysis, ++ between 20 and 50% lysis, + less than 20% lysis, - no lysis.

Cross-reactive clones maintain their cytolytic efficacy at lower peptide concentrations than serotype-specific clones.

CTL lysis assays were performed using B cells pulsed with serial dilutions (from 10μM to 0.001μM) of peptides representing the different dengue serotype variants of the A*11 GTS epitope. Consistent with the observations of clones derived from patient BC307, cross-reactive clones were able to lyse their targets at concentrations as low as 0.01μM. The DEN2 specific clones were not (Figure 36). All cross-reactive clones derived from MD1413 lysed B cells pulsed with the DEN2 variants of the epitope less effectively at moderate to low peptide concentration than those pulsed with the other variants.

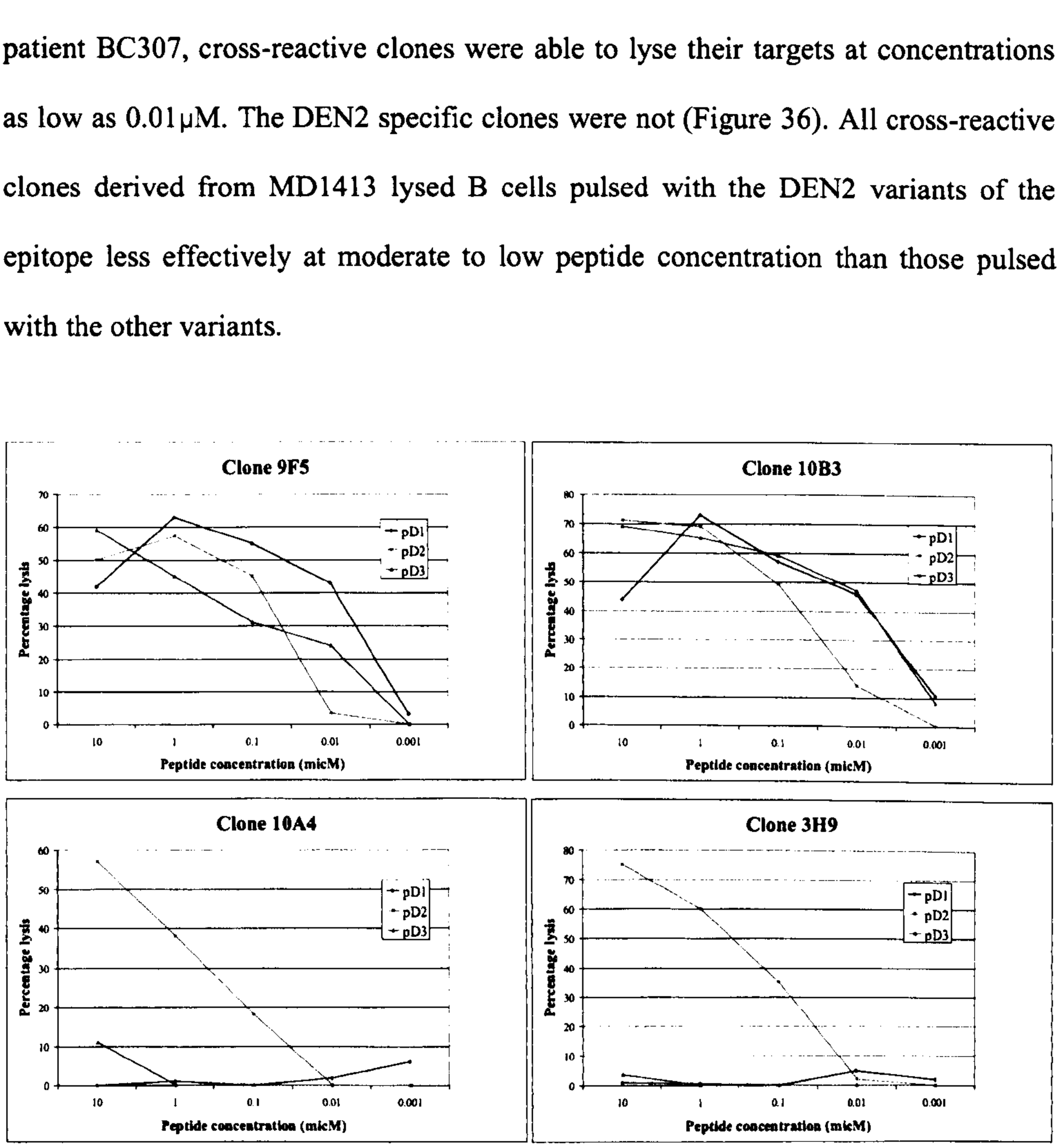
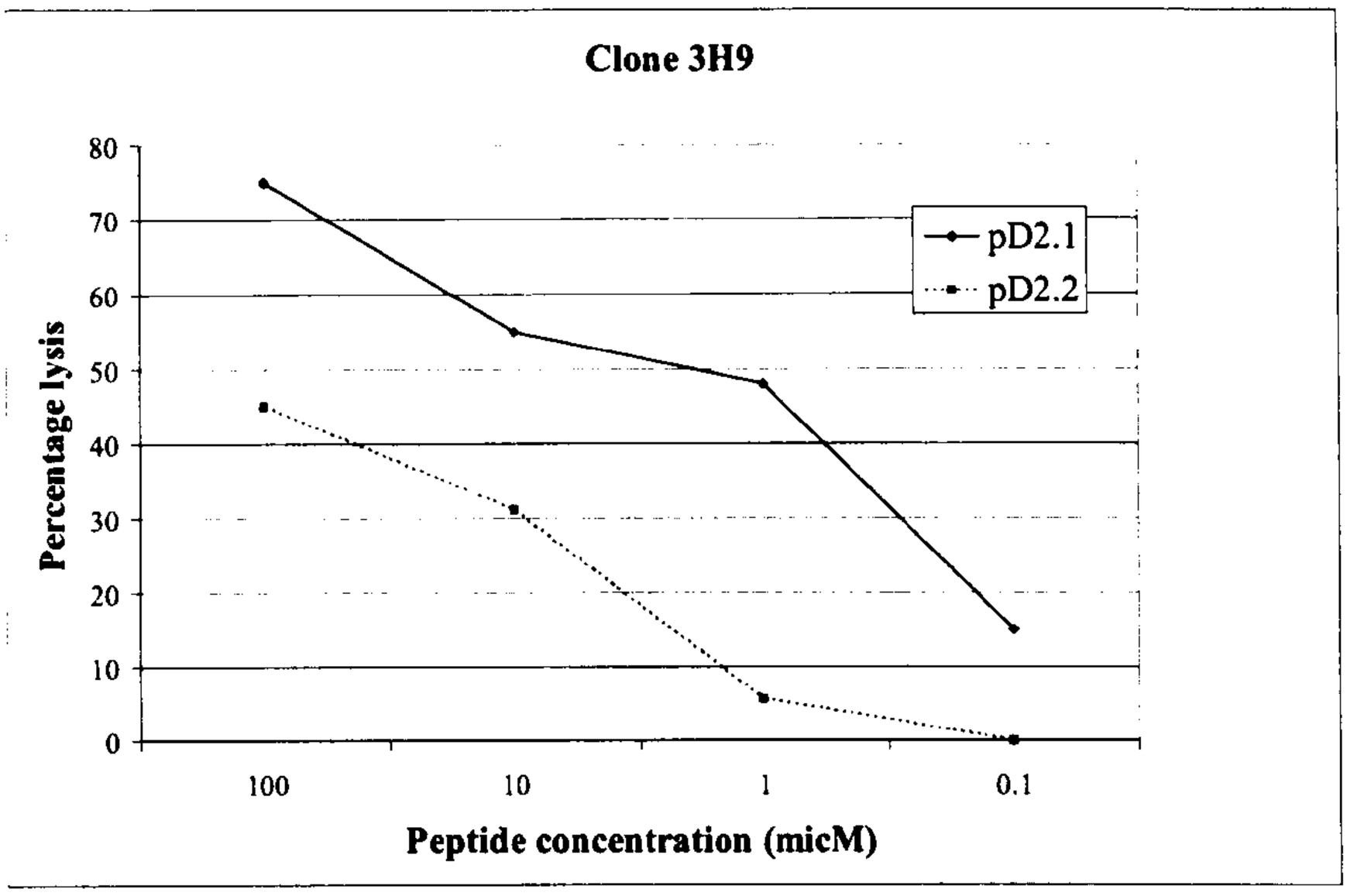


Figure 36. Clones differ in specificity and cytolytic efficacy in chromium-release assays. Cross reactive clones 9F5 and 10B3 maintain their cytolytic activity at concentrations as low as 0.01 and below. Dengue-2 specific clones 10A4 and 3H9 do not. Cells were used at an E:T ratio of 5:1.

DEN2 specific clones differed in their recognition of pD2 wild-type variants despite the same TCR Vβ usage.

Clones 3H9 and 10A4 – both specific for DEN2 – differed in their recognition of the wild-type variants of this epitope. 10A4 recognised pD2.2 (GTSGSPIVDK) only at very high peptide concentrations. 3H9 recognised pD2.2 to a greater extent but still lysed pD2.1 (GTSGSPIIDK) pulsed B cells much more effectively (Figure 37). The clones were stained with a panel of TCR Vβ antibodies. Both DEN2 specific clones were Vβ1 positive (Figure 38) suggesting that these more subtle differences in recognition were likely to be mediated by variations in the sequence of the CDR3 loop.



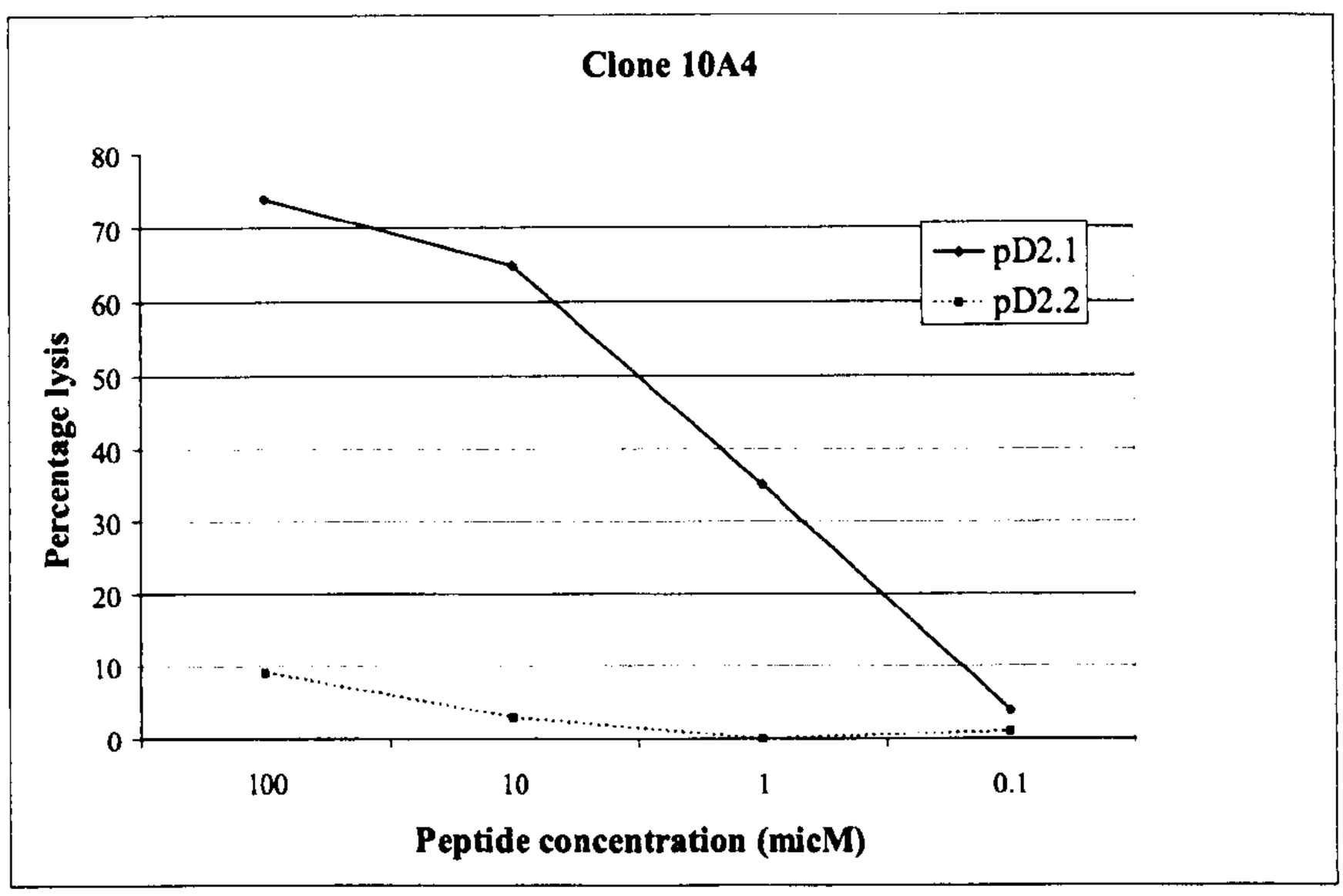


Figure 37. DEN2 specific clones 10A4 and 3H9 differ in their recognition of pD2 variants. Clones were incubated for 4 hours with chromium-51 A*1101 B cells pulsed with peptide for 1 hour at an E:T ratio of 5:1. pD2.1 sequence **GTSGSPIIDK**, pD2.2 sequence **GTSGSPIVDK**.

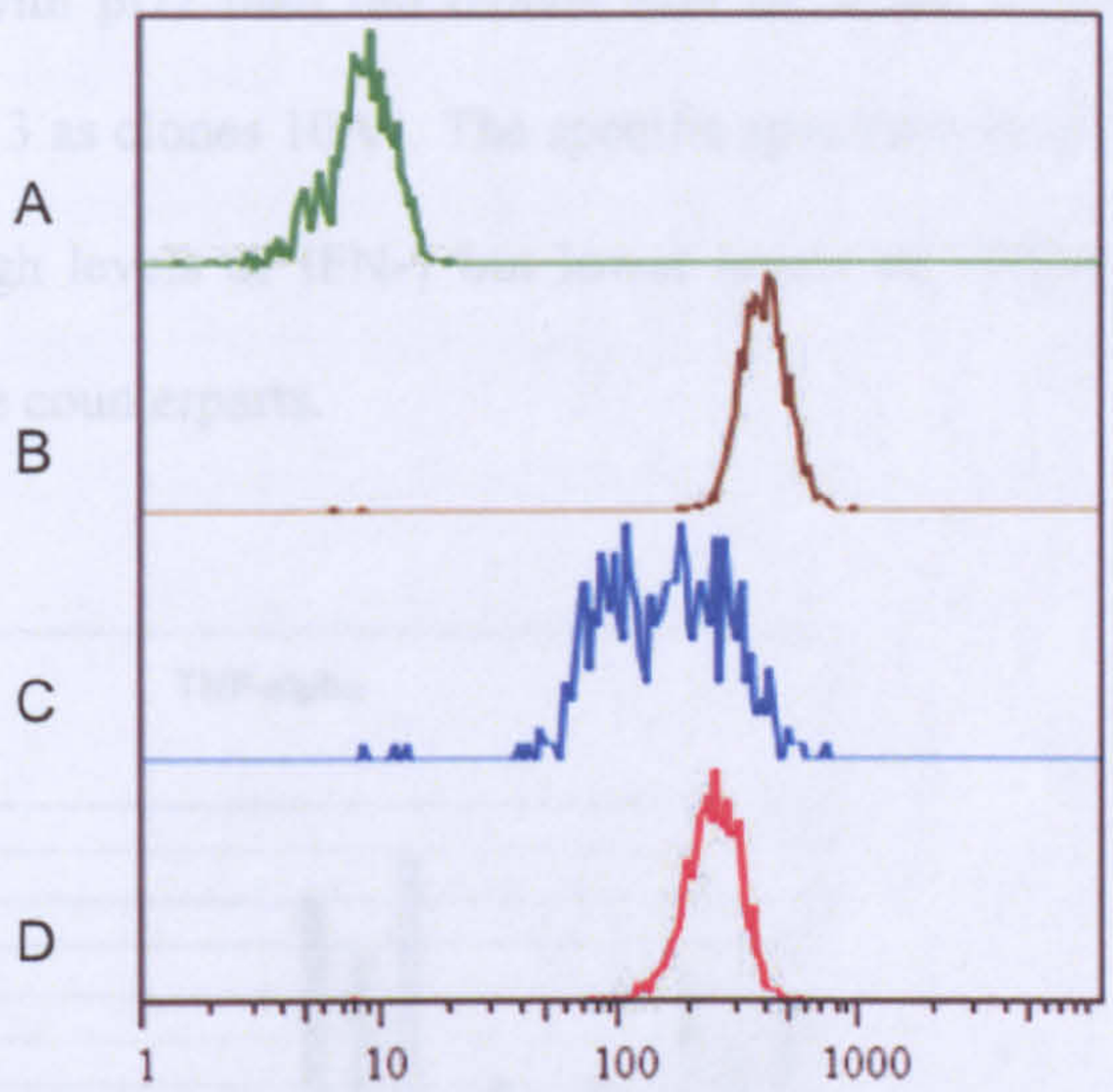


Figure 38. V beta antibody staining of clones. Sample A - negative staining of Vβ21.3 antibody with clone 10A4, Sample B, C and D – positive staining of clones 3H9 (Vβ1), 10H5 (Vβ21.3) and 10A4 (Vβ1) respectively.

Clones differ in the spectrum of cytokines they release and the amount in which each is produced.

Unlabelled B cells were pulsed with the GTS epitope peptide variants for 1 hour at 37°C and incubated with T cell clones overnight in a 96 well plate. The supernatant was then removed and the concentration of various cytokines within it measured using the Luminex system (see chapter 2). The clones differed in the spectrum and quantity of cytokines produced. Most fell into the type 0 category of cytokine producing T cells releasing both type 1 (e.g. TNF-α, IFN-γ) and type 2 cytokines (e.g. IL-4, IL-13). Unlike the earlier work described above IL-10 was not produced in significant amounts. However, consistent with these observations cross-reactive clones generally produced much higher levels of most cytokines than the serotype-specific clones for a given stimulation (Figure 39). For example 10H5 produced 6 times as much TNF-α

when stimulated with pD2 than did clones 3H9 or 10A4. Clone 9F5 produced 20 times as much IL-13 as clones 10A4. The specific spectrum varied: for example clone 10B3 produced high levels of IFN-γ but lower levels of TNF-α and IL-13 than its other cross-reactive counterparts.

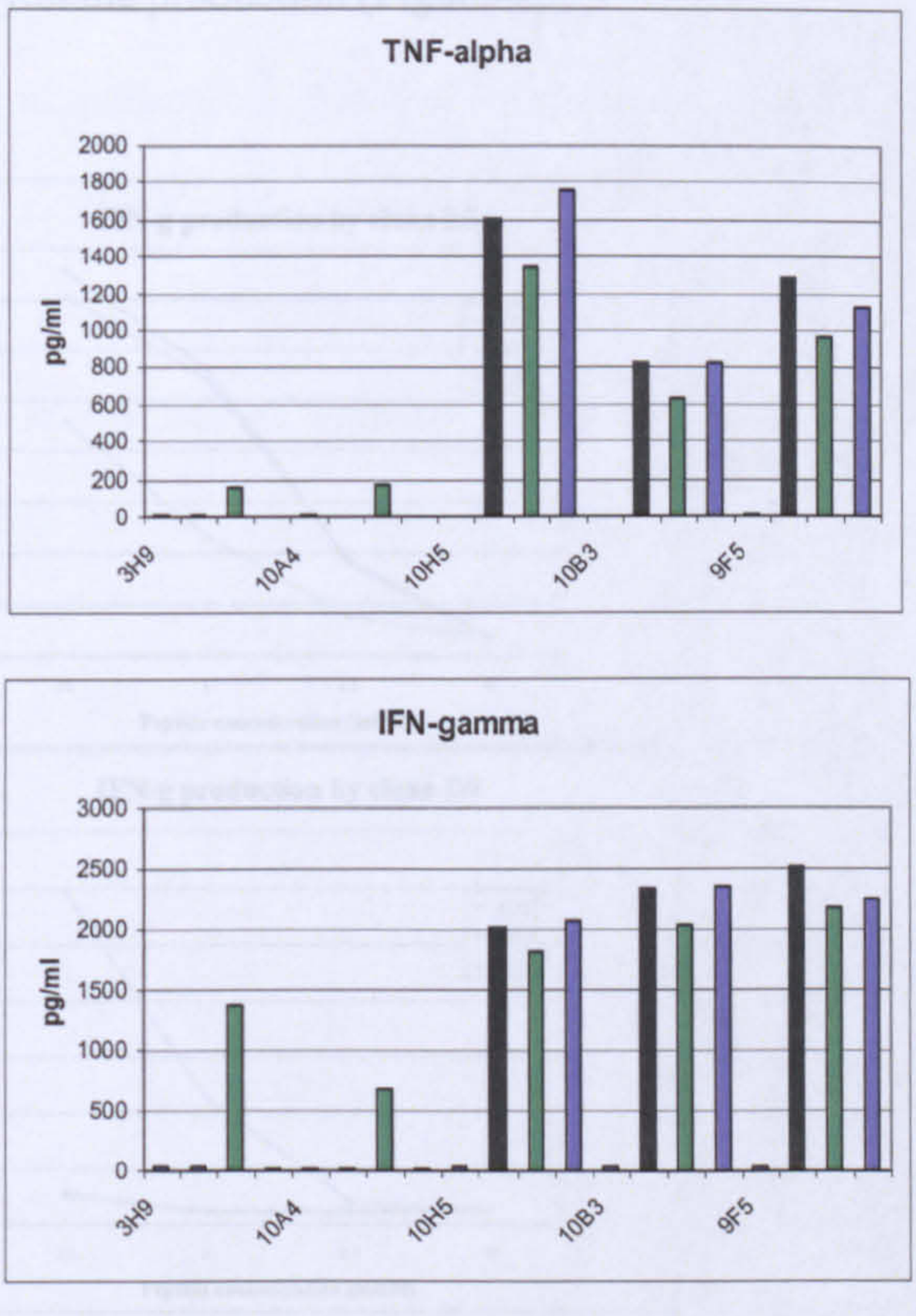


Figure 39. Clones from patient MD1413 differ in the spectrum and levels of cytokines produced. A*1101 B cells were pulsed with peptide for 1 hour at 37°C and incubated with clones at an E:T ratio of 5:1 overnight in 150µl H10. 70µl of the supernatant was removed and used for cytokine analysis on the Luminex system.

Cytokine release with titrations of the stimulating peptide was performed with clones E5 (cross-reactive) and D9 (dengue 3 specific) derived from patient BC307 (supplied by Dr Dong). The pattern of cytokine production closely resembled that seen in the CTL lysis assays but lytic ability was maintained at lower peptide concentrations than was cytokine production (Figure 40).

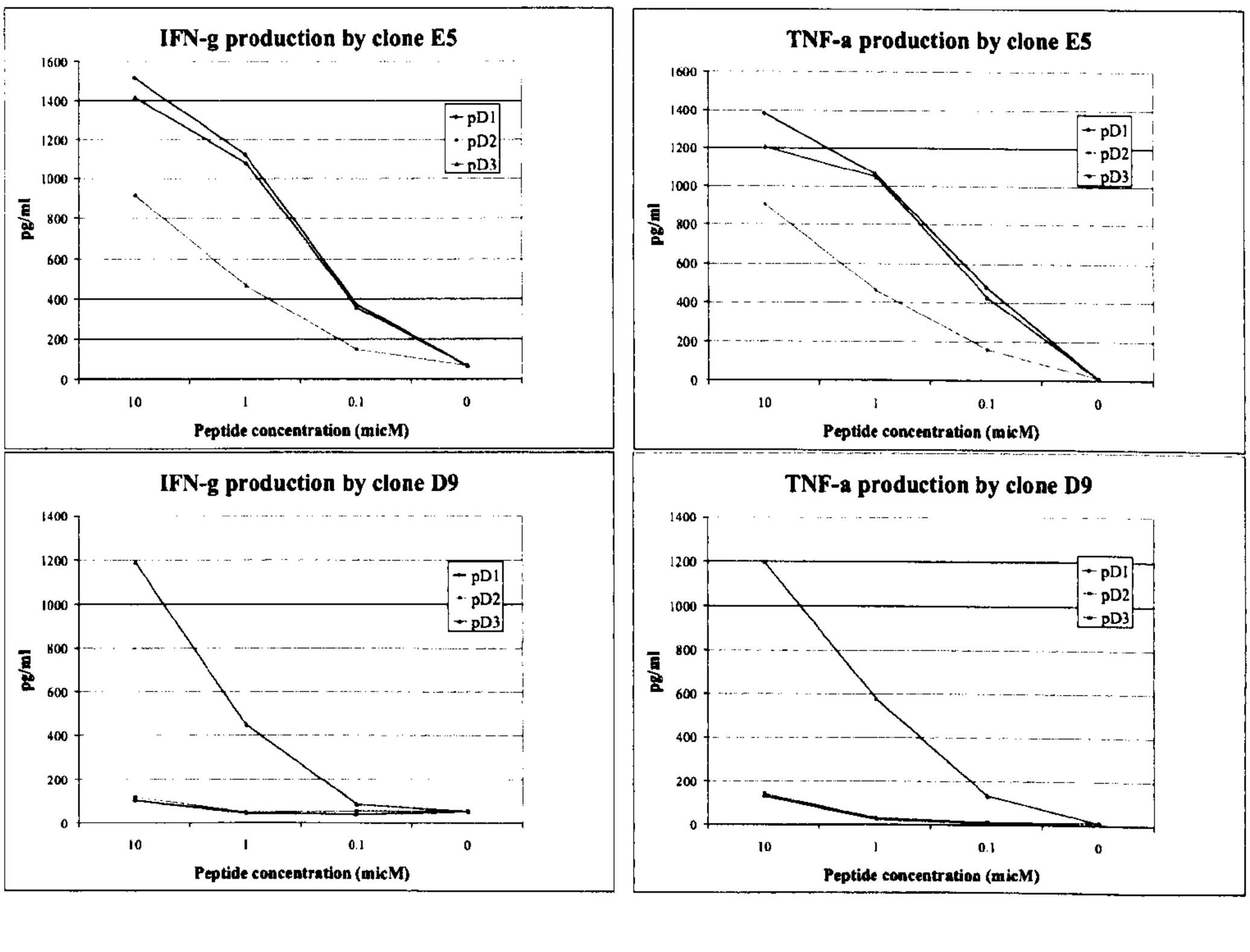
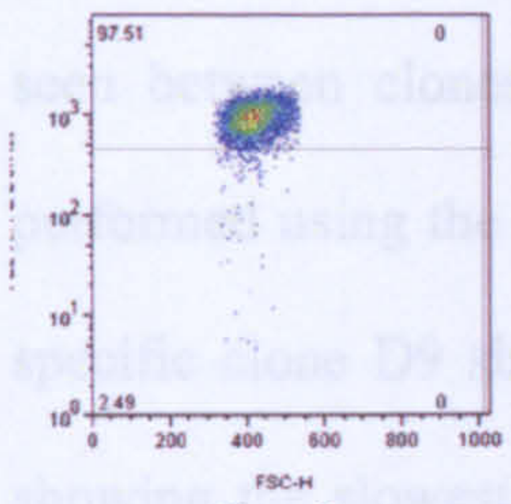


Figure 40. IFN-γ and TNF-α production by clones E5 and D9 at an E:T ratio of 5:1
Both produce similar quantities when stimulated by cognate peptide at high concentrations. At lower concentrations cross-reactive clone E5 produces up to 4 times more IFN-γ than serotype specific D9. Cytokine production drops off faster than a clone's lytic efficacy at low peptide concentration. This figure is representative of three independent experiments.

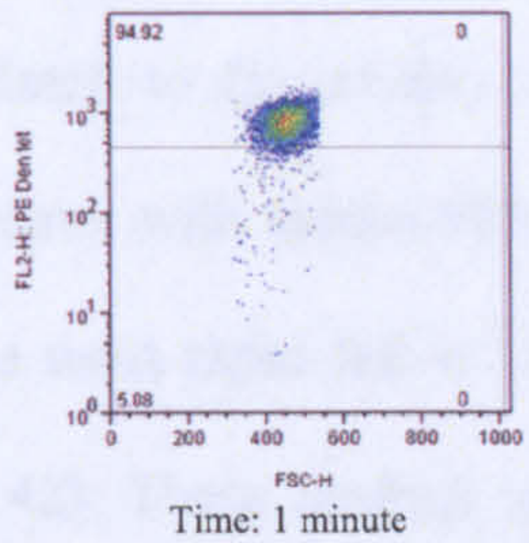
Cross-reactive clones tend to show a greater avidity for tetramer than serotype-specific clones in tetramer decay assays.

Tetramer decay assays give an indication of the relative avidity of two or more clones for a given tetramer. Clones are stained with tetramer but rather than fixing are then incubated with an excess of a competing ligand – either the same tetramer bound to a different marker (e.g. APC) or an antibody capable of blocking tetramer rebinding. As the original tetramer vacates the TCR its place is taken by the alternative ligand and it cannot rebind. Comparing the fall in fluorescence intensity between clones allows a crude assessment of relative avidity (see chapter 2 for a full description of the method). An example plot can be seen in Figure 41.

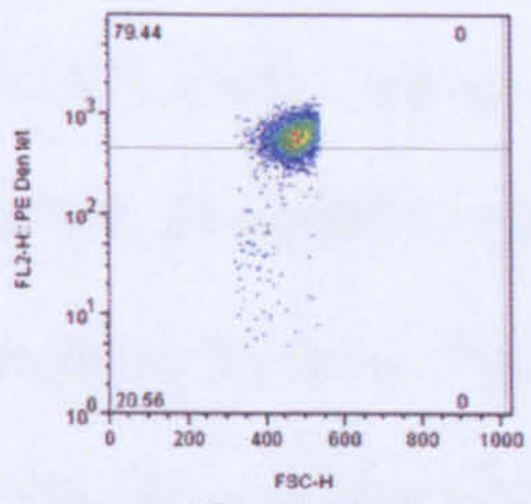
A



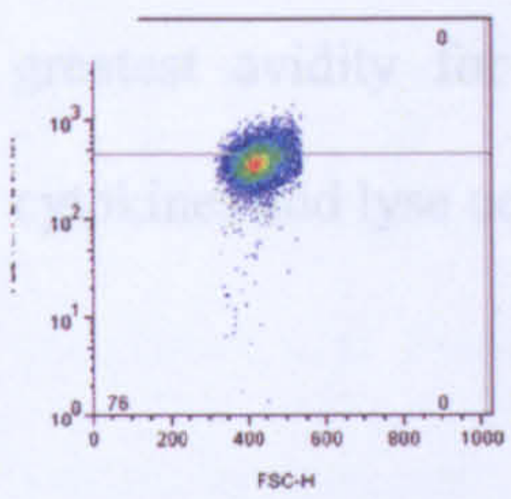
Time: 0



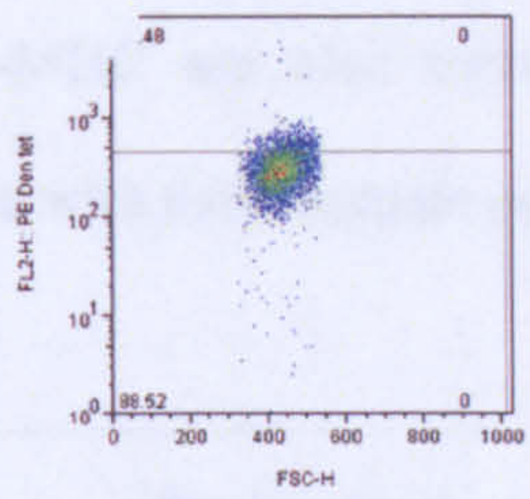
Time: 1 minute



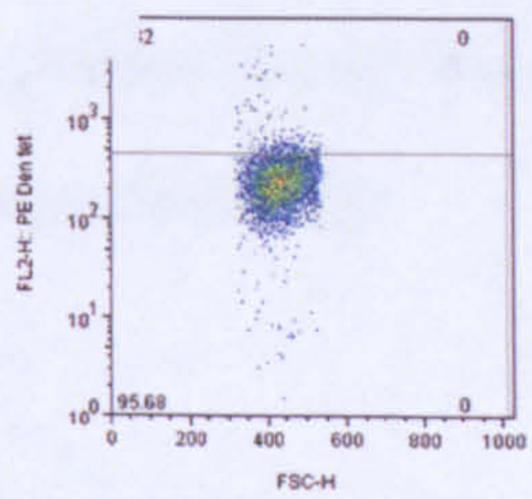
Time: 5 minutes



Time: 15 minutes



Time: 30 minutes



Time: 60 minutes

B

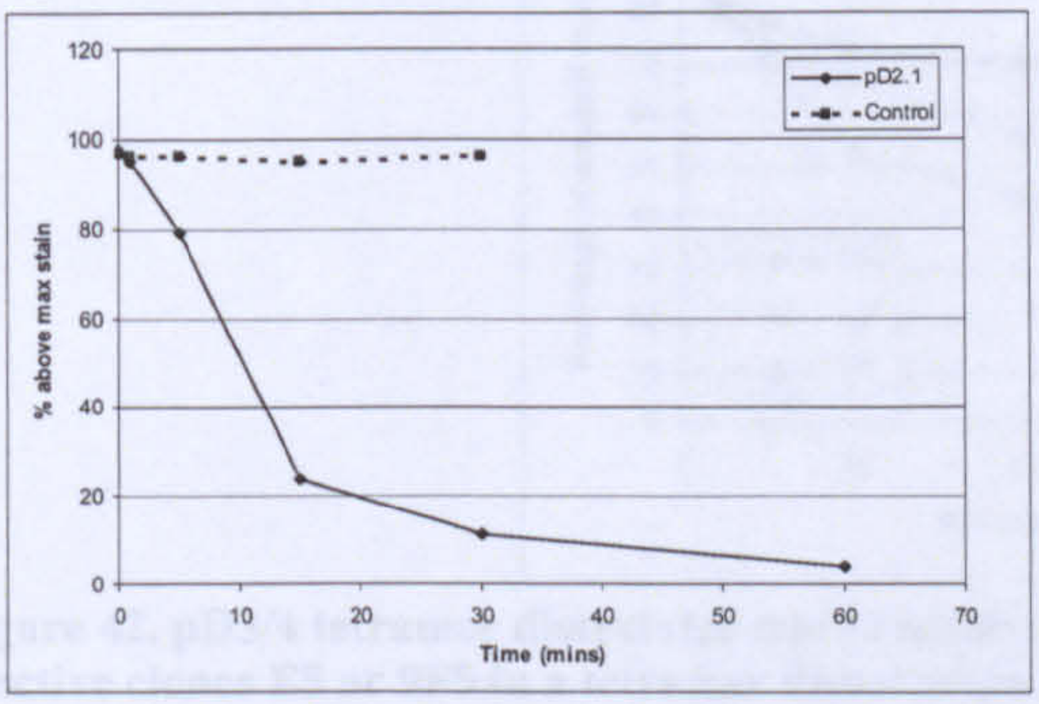


Figure 41. Decay assay for clone 9F5 stained with pD2 tetramer. Panel A: Serial FACS plots. The reference intensity in this example is that at above which 97% of cells are found at time 0. The number remaining above this line as time passes is recorded. In the absence of a competing ligand the cell number remains stable above 90% for the duration of the assay. Plots are gated on lymphocytes. The FACS plots show the data for pD2 PE-tetramer labelled 9F5 competing with an excess of pD2 APC-tetramer. **Panel B:** Time course of fluorescent intensity. The same data shown in a chart allowing comparison with a negative control (pD2 PE-tetramer stained 9F5 cells with no competing ligand present).

Conditions for this assay: 1 million cells of clone 9F5 were stained with 2µl of pD2.1 tetramer (equivalent to 1.13µg) for 45 minutes at 4°C. After washing twice in FACS buffer and resuspending in a total volume of 40µl PBS, 2µl of the reaction was taken and mixed with 200µl of PBS and analysed on a FACS machine. The reaction was then mixed with either APC conjuagted tetramer at a concentration 10 times that originally used to stain the cells or the same volume of FACS buffer as a negative control. 2-4µl of the reaction was taken at the indicated times, mixed with 200µl of PBS and analysed.

To determine whether the difference in cytolytic behaviour and cytokine production seen between clones was related to the avidity of their TCR decay assays were performed using the pD3 tetramer with clones 9F5, E5 and D9. The DEN3 serotype specific clone D9 showed the most rapid fall of in fluorescence intensity with 9F5 showing the slowest (Figure 42). These findings suggest that those clones with the greatest avidity for peptide-MHC are also those that produce higher levels of cytokines and lyse cells loaded with their cognate peptide most effectively.

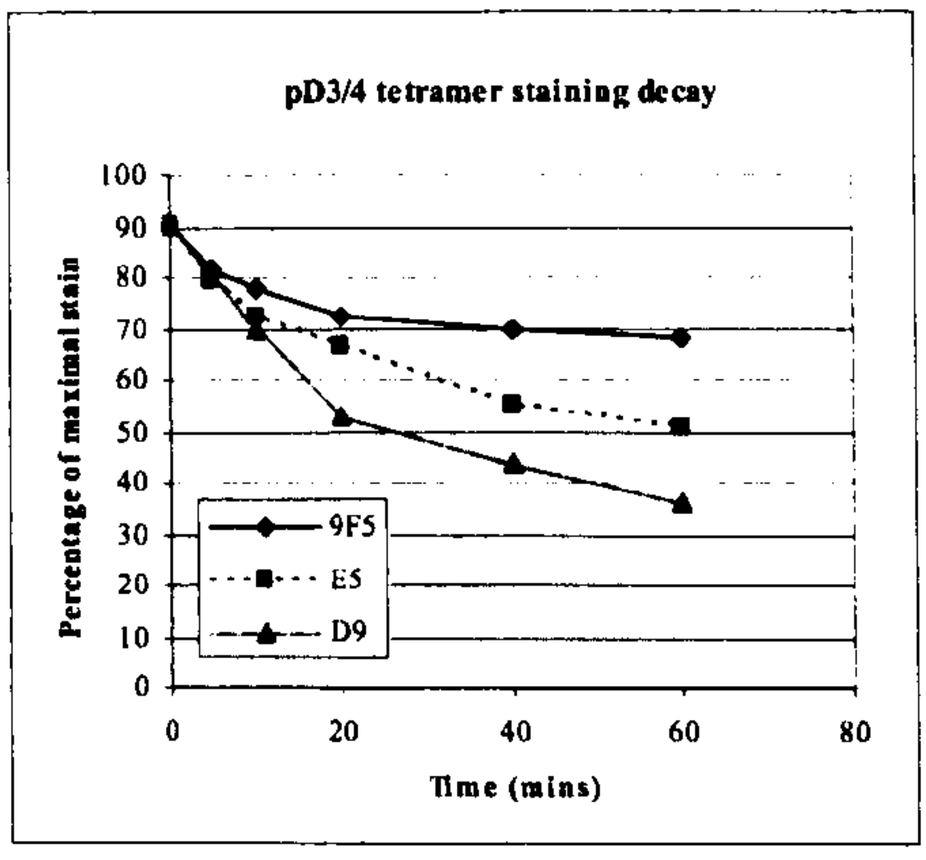


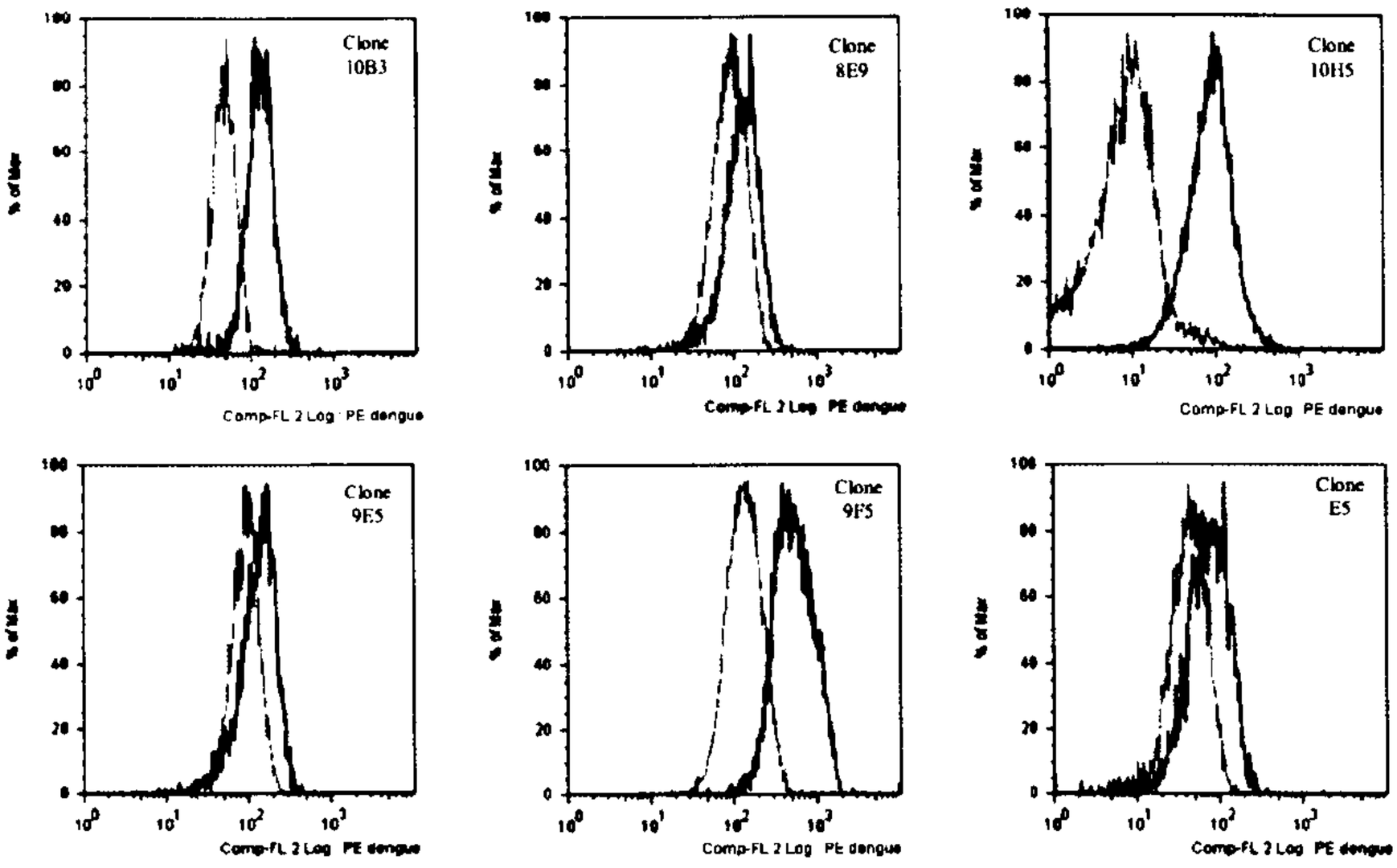
Figure 42. pD3/4 tetramer dissociates more rapidly from serotype specific clone D9 than cross-reactive clones E5 or 9F5 in a tetramer dissociation assay. Performed as described above.

Cross-reactive clones show greater binding to CD8-null tetramers than serotype-specific clones.

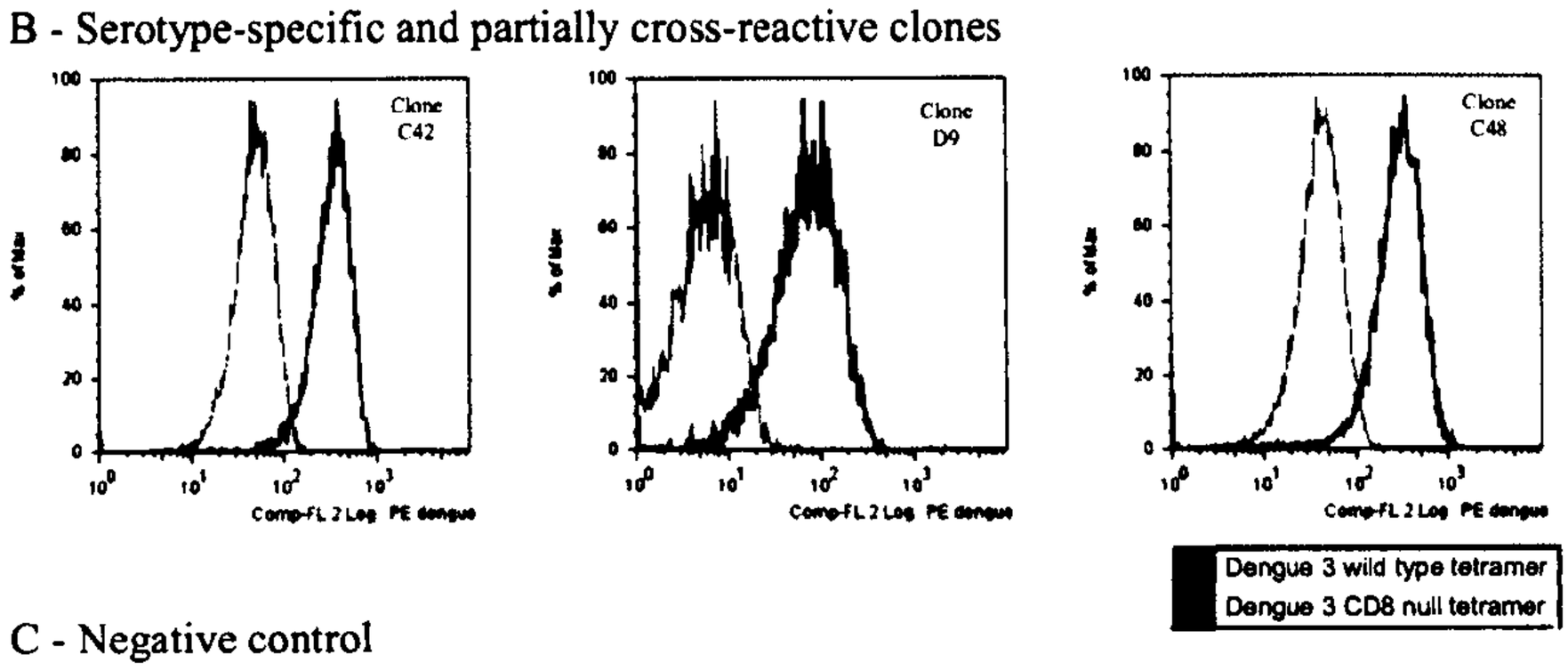
Tetramers were produced that were incapable of binding CD8 due to a mutation in the α3 region of the heavy chain (see above). Such tetramers have been shown to reliably identify high avidity CTLs (241). Clones derived from both patient MD1413 and BC307 were stained with these "CD8-null" tetramers folded with pD3/4. Cross-reactive clones 9F5 and E5 bound this tetramer almost as well as the wild-type tetramer (Figure 43 – panel A) whereas more serotype specific clones C48 and D9

showed little or no binding (Figure 43 – panel B). Other cross-reactive clones not described here in detail showed similarly effective binding to the CD8-null tetramer (8E9, 9E5). This implies that cross-reactive clones are less reliant upon the CD8 interaction for binding than serotype specific clones or those with only low level cross-reactivity, perhaps reflecting a stronger peptide/MHC-TCR interaction. The relative weakness of the peptide/MHC-TCR interaction of a serotype specific clone renders the MHC-CD8 interaction a requirement for tetramer staining. This is consistent with those observations made by tetramer dissociation assays. D9's TCR avidity for tetramer is lower than its cross-reactive counterparts.

A - Highly cross reactive clones



B - Serotype-specific and partially cross-reactive clones



C - Negative control

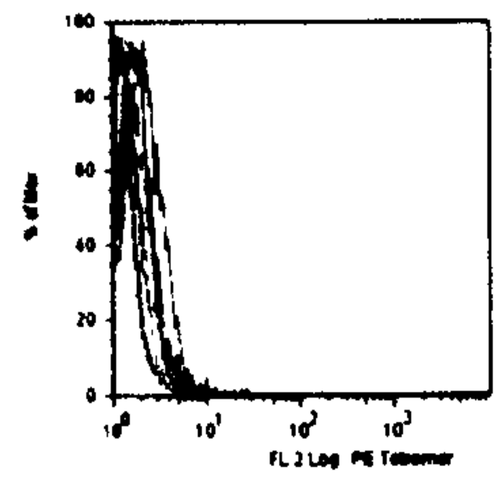


Figure 43. pD3/4 CD8-null tetramer binds cross-reactive clones more effectively (A) than serotype specific or partially cross-reactive clones (B). Figure C: Negative control - none of the clones showed any significant binding to an irrelevant tetramer.

All clones show lower avidity for the pD2 epitope variant.

None of the clones generated from these patients, whether cross-reactive or dengue 2 specific, showed any significant binding to the pD2 CD8-null tetramer (Figure 44 –

panel A). Dissociation assays demonstrated that pD2 wild-type tetramer dissociates from cross-reactive clones much more quickly than pD3/4 (Figure 44 – panel B). These observations fit the phenotypic differences noted earlier: cross-reactive clones tended to produce slightly less of each cytokine, and lyse less effectively when stimulated by pD2 at lower concentrations. pD2 conforms to published A*1101 binding motifs. It would appear that effective TCR binding is dependent upon the MHC-CD8 interaction to an extent not demonstrated by the other variants, suggesting that the pD2-MHC interaction with TCR is of inherently lower avidity than the other variants. The differences between pD2 and the other 2 variants involve residues highly likely to be involved in anchoring.

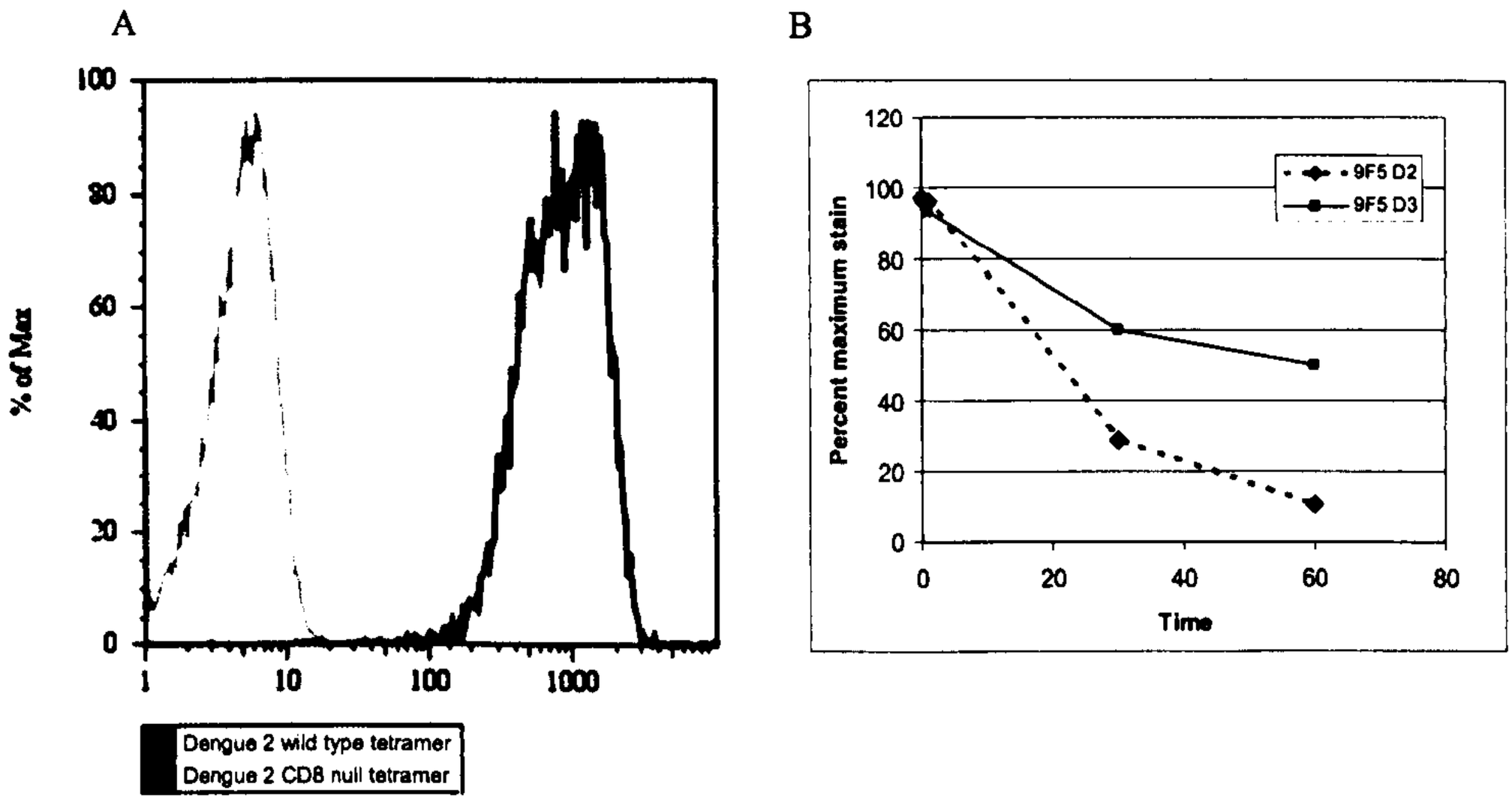


Figure 44. pD2 CD8-null tetramer failed to bind any clone and pD2 wild-type tetramer binds cross-reactive clones more weakly than either pD3/4 or pD1. A) Clone 9F5 stained with wild-type (green) and CD8-null versions of the pD2 tetramer. B) Decay assay comparing the relative fall in maximum stain between clone 9F5 stained with wild-type pD2 and pD3/4 tetramers.

It was not possible to determine whether the high-affinity tetramer positive fraction falls between acute disease and convalescence.

Frozen acute and convalescent PBMC from 8 A*11+ dengue patients and 9 B*07+ dengue patients were thawed and stained with CD38 antibody, CD8 antibody, CD4 antibody and the appropriate dengue tetramer in either its wild-type or CD8-non-binding form. The fraction of wild-type tetramer positive cells that stained with the CD8-non-binding tetramer in acute disease was compared with that in convalescence. The majority of acute samples showed negligible tetramer staining. Most cells in these samples were dead. This may reflect the highly activated nature of lymphocytes in acute disease or perhaps the time taken to cryopreserve the samples in the field. Just one A*11 patient had good acute and convalescent staining (DF135, with DHF III). 33% of the total A*11 tetramer positive fraction stained with the CD8-non-binding tetramer in acute disease compared to 30% in convalescence (Figure 45). This fall is too small to be of significance and these observations need to be repeated in a large number of samples if any conclusion is to be drawn. All patients showed high levels of CD38 expression acutely in both the CD8+ and CD4+ subsets, which had reduced by convalescence (see Figure 46 from representative patient DF133).

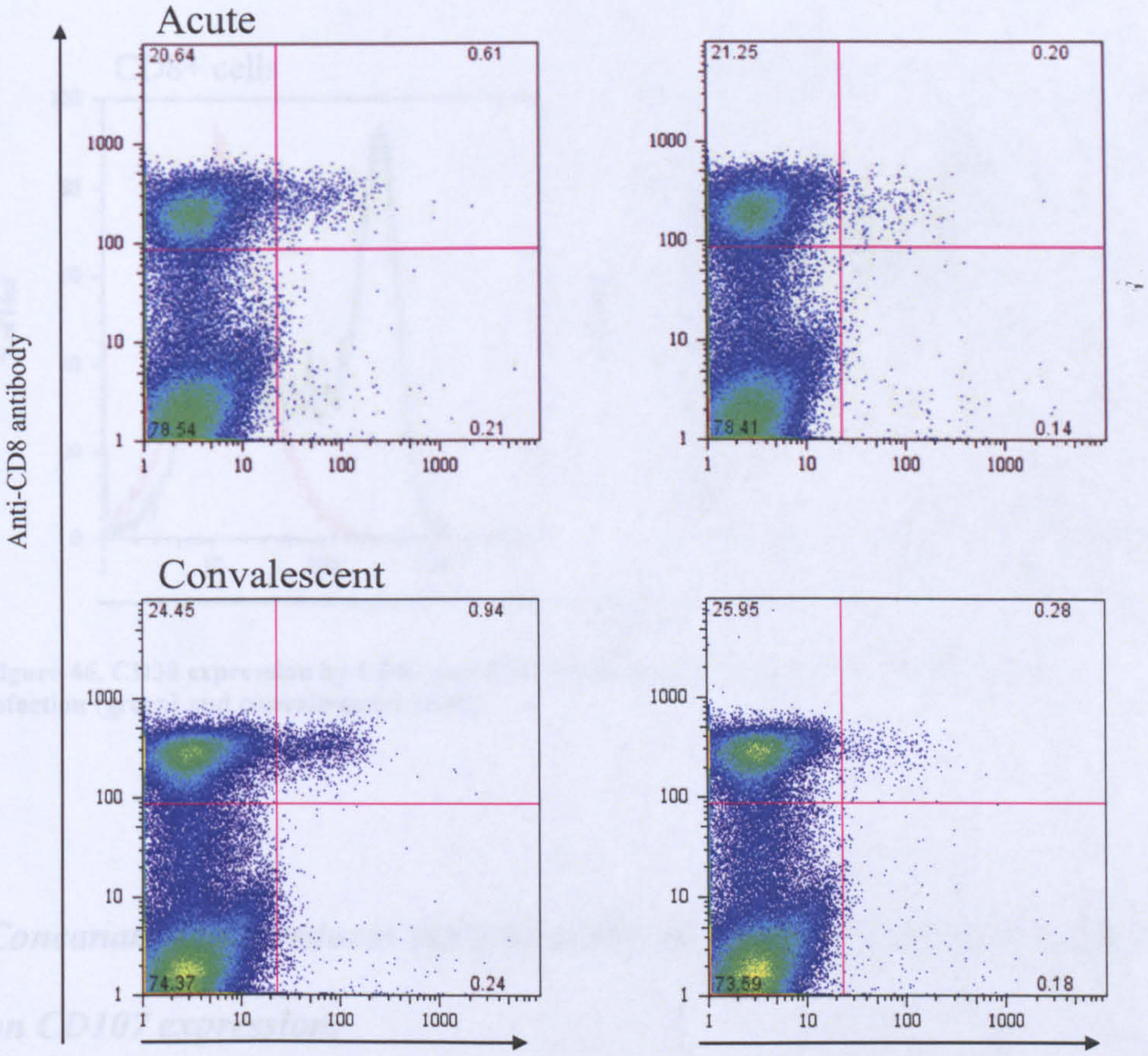


Figure 45. The fraction of A*11 dengue 3/4 tetramer positive cells that stain with CD8-non-binding tetramer falls from 33% in acute infection to 30% in convalescence (patient DF135). The top panels show tetramer staining of a PBMC sample taken on day 2 of infection. The lower panels show identical staining of a sample taken 32 days later. Left hand side: wild-type D3/4 tetramer; Right hand side: CD8-non-binding D3/4 tetramer.

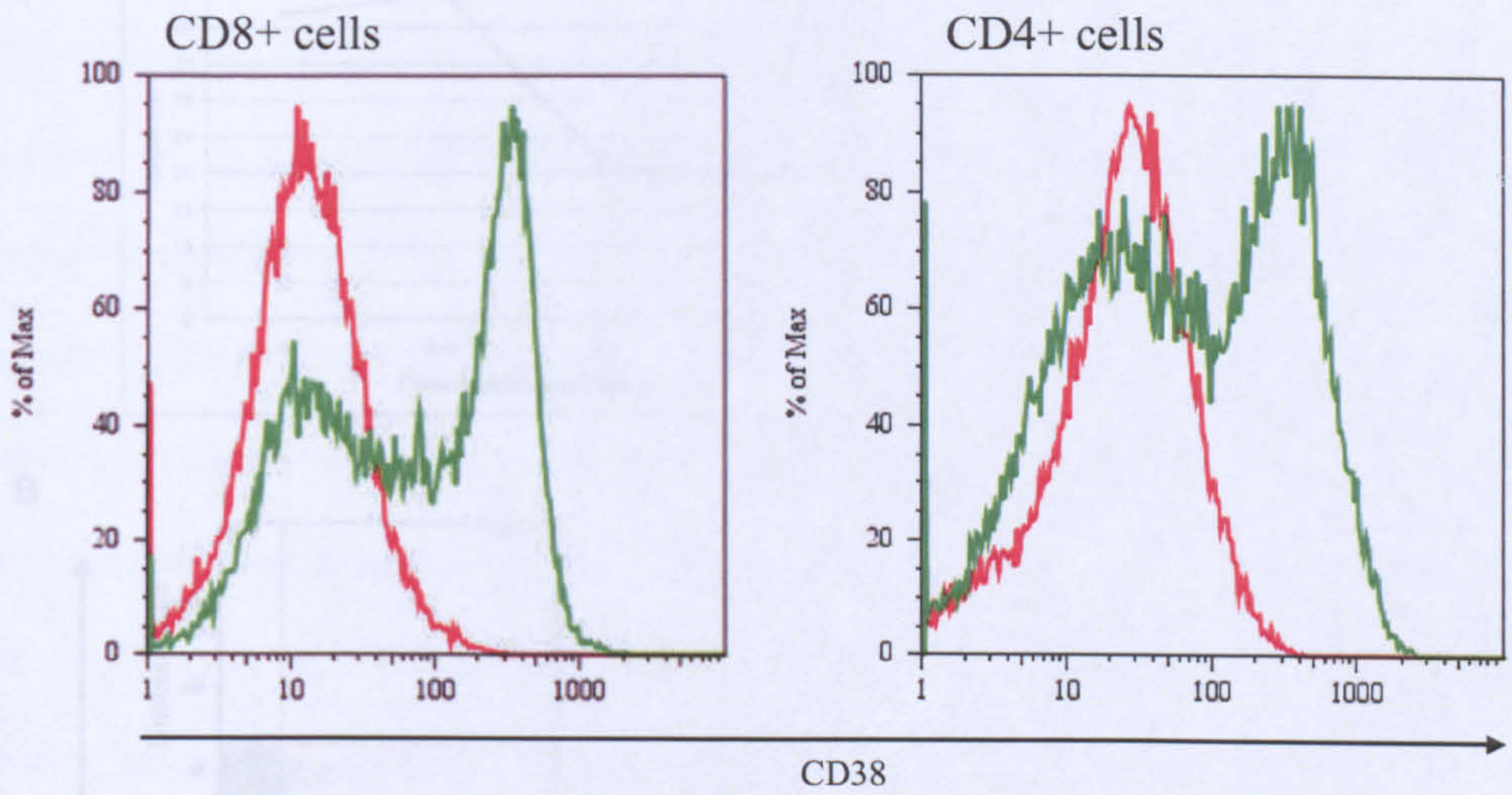


Figure 46. CD38 expression by CD8+ and CD4+ lymphocytes from patient DF133 in acute infection (green) and convalescence (red).

Concanamycin A reduces the lytic ability of clone 9F5 but has no effect on CD107 expression.

Concanamycin A (ConA – Sigma) inhibits perforin mediated killing by enhancing its degradation within granules. 50000 cells of clone 9F5 were incubated with ConA at concentrations of either 1μM, 0.1μM or 0.01μM for 1 hour at 37°C before using in a standard CTL lysis assay with A*11 B cells pulsed with pD3/4 (10μM) at an E:T ratio of 10:1. The addition of ConA brought about a reduction in specific lysis (Figure 47). Cells of clone 9F5 were incubated with either 1μM of ConA or 10μM colchicine for 1 hour before stimulating with pD3/4 pulsed B cells in the presence of CD107 antibody (a marker of degranulation – see chapter 5) as described in chapter 2. CD107 expression was not affected by the presence of ConA. It was however reduced by the presence of colchicine (figure 47 – panel B) – an inhibitor of microtubule function.

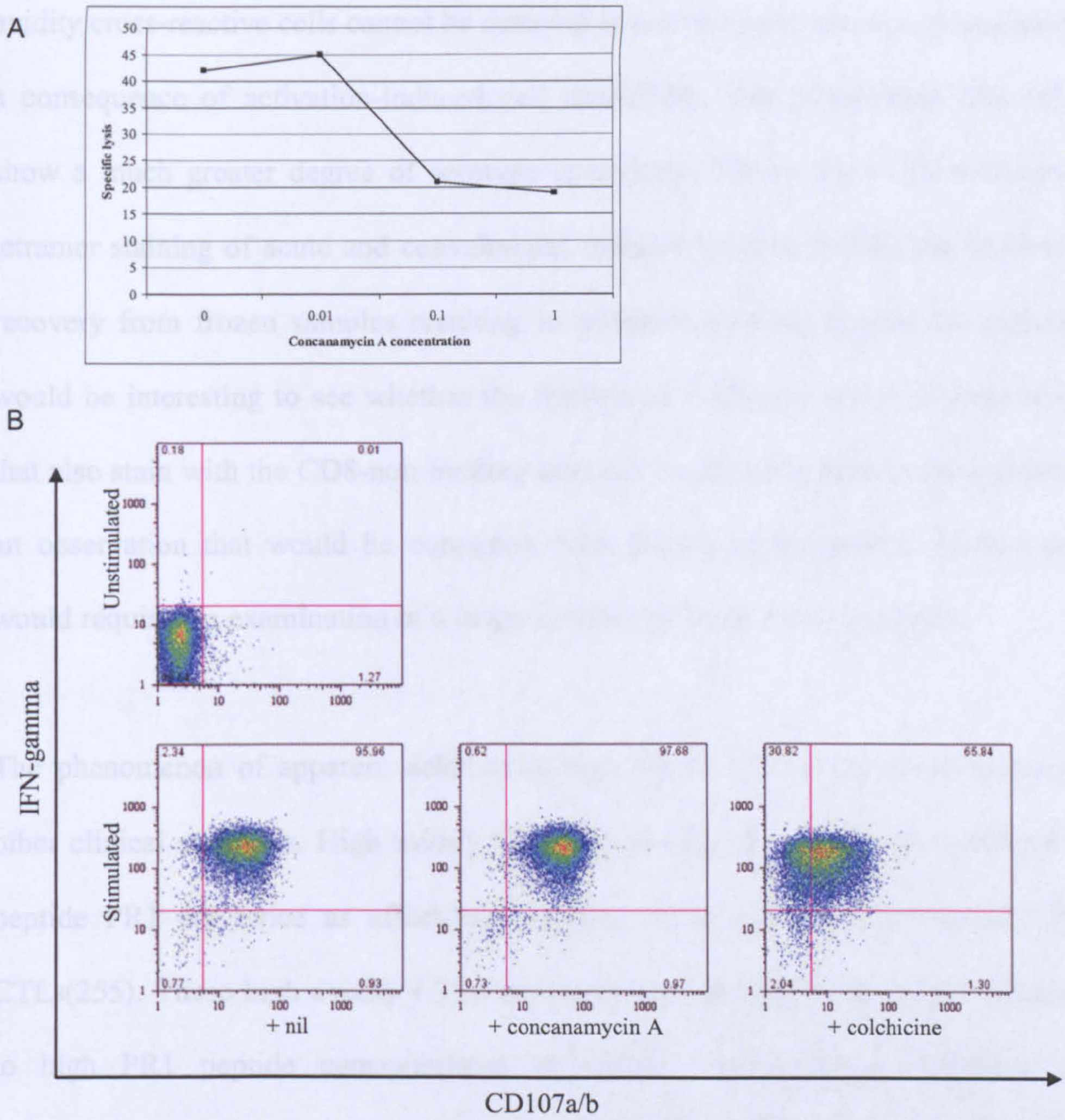


Figure 47. The addition of concanamycin A to clone 9F5 eliminates cytolysis but does not reduce degranulation marker CD107ab upregulation. A) Effect of preincubation of T cells with differing concentrations of ConA on a standard CTL lysis assay. B) Effect of preincubation of T cells with ConA (1μM) or colchicine (20μM) on CD107 surface expression. 100000 A*11 B cells (unpulsed or previously pulsed with 20μM pD3/4) were incubated with 100000 cells of clone 9F5 in the presence of anti-CD107a and CD107b FITC. After 1 hour monensin was added and after 5 hours the cells were washed, permeabilised and stained with IFN-γ APC and CD8 PerCP. Analysis was conducted after gating on lymphocytes.

Discussion

This study demonstrates that a population of activated high avidity CTLs showing substantial cross-reactivity between all four dengue serotypes can be detected in certain hospitalised Vietnamese patients with acute dengue virus infection. These high

avidity cross-reactive cells cannot be detected in convalescent samples, presumably as a consequence of activation-induced cell death(254). The populations that remain show a much greater degree of serotype specificity. The *ex vivo* CD8-non-binding tetramer staining of acute and convalescent samples is inconclusive, the limited cell recovery from frozen samples resulting in adequate staining in just one patient. It would be interesting to see whether the fraction of wild-type tetramer positive cells that also stain with the CD8-non-binding tetramer consistently falls in convalescence, an observation that would be consistent with the above hypothesis. Such a study would require the examination of a larger number of **fresh** *ex vivo* samples.

The phenomenon of apparent deletion of high avidity CTLs has been observed in other clinical scenarios. High avidity CTL specific for the leukaemia-associated self peptide PR1 are twice as effective at killing CML cells than low-avidity PR1-CTLs(255). These high avidity CTLs are selectively deleted by apoptosis if exposed to high PR1 peptide concentrations or chronic myelogenous leukaemia cells overexpressing proteinase 3 (the protein from which the PR1 epitope is derived). The investigators were able to expand or detect both low and high avidity PR1-CTLs from healthy donors, but only low avidity CTLs could be expanded or detected from newly diagnosed leukaemia patients(255). The authors suggest that this process of selective clonal deletion may be a result of clonal exhaustion in a manner similar to that observed in LCMV infection(256). Outside the clinical setting it has also been observed that CTL are susceptible to proliferative inhibition by high dose peptide antigen, leading to apoptotic death(252). It has been noted previously that tetramer positive lymphocytes from patients with acute dengue are proliferating and dying in

large numbers (189). This may reflect the high avidity shown by many cross-reactive cells for their targets and the large antigen load found just before defervescence.

Why cross-reactive cells would be more likely to be of high avidity is not clear. TCR cross-reactivity is mediated primarily by the structure and flexibility of the CDR3 region whereas TCR avidity reflects the molecular "fit" between peptide-MHC and TCR pocket. Functional T cell avidity reflects the sum of several factors (discussed below p.148) and can be defined as the concentration of peptide that leads to half the maximal activation of T cells in a given population for a constant number of antigen presenting cells – thus highly avid cells achieve the same level of activation with much lower peptide concentrations than required for low avidity cells(257). Wilson et al(258) suggest that clones of high avidity should demonstrate low levels of cross-reactivity. They argue that "a large but finite … peptide universe should, in general, contain fewer sequences capable of stimulating a high avidity T cell clone i.e., one with a higher threshold of activation, than a lower avidity, comparatively more degenerate clone."[1] These authors use the term "dengeneracy" interchangeably with "cross-reactive". Some sources include those cells capable of recognising different **MHC** molecules within the term "degenerate T cells" (259) but for the purposes of this discussion "cross-reactivity" is defined as the capacity of certain TCRs to recognise different epitopes presented by the same MHC molecule. To paraphrase Wilson's thinking: of all available peptides in the world very few will exist capable of

[1] An argument with more than a passing resemblance to Douglas Adams' conclusion that the population of the universe is zero: "It is known that there are an infinite number of worlds, simply because there is an infinite amount of space for them to be in it. However, not every one of them is inhabited. Therefore, there must be a finite number of inhabited worlds. Any finite number divided by infinity is as near to nothing as makes no odds, so the average population of all the planets in the Universe can be said to be zero. From this it follows that the population of the whole Universe is also zero, and that any people you may meet from time to time are merely the products of a deranged imagination." Adams, D. *The Hitchhikers Guide to the Galaxy.*

binding a "high avidity" T cell clone as effectively ("avidly") as its "primary" ligand and so attain the cell's activation threshold. This conclusion fits well with their observation that highly avid T cell clones recognise fewer peptides from a large random peptide library than lower avidity clones. It is however difficult to use the same reasoning in the setting of clinical dengue. The peptides we are comparing are closely related, differing by only one or two amino acids (DK to NR) which possess similar physical properties (e.g. all are hydrophilic and tend to form H bonds, both K and R at position 10 of the epitope are basic). Perhaps a different argument applies in such settings and it is precisely because of the strength of its interaction with p-MHC that a TCR showing high avidity is able to tolerate small changes in the peptide ligand sequence, hence demonstrating cross-reactivity. Something *is* lost however in these changes: it is clear from figure 43 that the avidity cross-reactive clones exhibit for pD2 tetramers is lower than that exhibited for the others. The free amino and carboxyl termini of an epitope play a key role in class I MHC binding(100) and it seems likely that the relatively undramatic differences (in terms of the physical properties of the amino acids concerned) between pD3/4 and pD2 would have a minimal impact on epitope binding to HLA A*11. It is possible that these amino acids are important in TCR recognition. It has been theorised that the CD8 molecule enhances the affinity of TCR for its ligand by reducing the "off" rate, guiding an energetically favourable docking of TCR onto MHC and possibly by inducing conformational changes in the MHC complex that serve to augment the TCR/MHC-peptide interaction(244). Such mechanisms might explain why pD2, a peptide apparently utterly incapable of binding TCR when complexed with CD8-non-binding tetramers, can nonetheless act as effective antigen in complex with "wild-type" HLA A*11.

That the cross-reactivity/avidity relationship observed here may simply be artefactual cannot be excluded. Most clones generated were cross-reactive to a greater or lesser extent and only 3 were truly serotype-specific. It would appear that most TCRs within an infected individual are able to bind to all variants of the A11 GTS epitope to some extent and relatively few are discriminating enough to recognise only one serotype. Further work attempting to generate more unique serotype-specific clones would be necessary before one could conclude that such CTLs were consistently of lower avidity than their cross-reactive counterparts.

All the clones in this study produced both type 1 and type 2 cytokines: IFN-γ, TNF-α, IL-4, IL-13 and in some cases IL-10. TNF-α can mediate activation-induced cell death in some T cells (260) and has been implicated in peripheral T cell deletion (261, 262). Type 2 cytokines such as IL-13 and IL-10 have been implicated in the pathogenesis of severe dengue(88, 90). IL-10 can be produced by distinct CD4+ and CD8+ T cell populations and has the ability to suppress T cell function (263, 264). It could be postulated that certain type 2 cytokines produced in acute disease by a subset of highly cross-reactive CTL might exert an inhibitory effect on dengue specific effector T cells. Highly cross-reactive clones grown from acute samples produced high levels of TNF-α, occasionally IL-10 and demonstrated the greatest avidity for peptide-MHC as demonstrated by staining with CD8-null tetramers and tetramer decay assays. Serotype specific and partially cross-reactive clones produced much less TNF-α, IFN-γ and GM-CSF and were not shown to secrete any IL-10. It has been suggested that the pattern of CD8+ cell cytokine production is epitope dependent (265, 266) with high avidity T cell/target interactions leading to greater production of TNF-α or IFN-γ (267-269).

In general killing ability correlated well with the TCR/pMHC avidity demonstrated by CD8-null tetramers. There were variations in TCR avidity amongst clones that nonetheless displayed very similar levels of cytolytic activity for a given peptide concentration. For example the lytic activity of highly cross-reactive clone E5 was very similar to partially cross-reactive clone C48 for a given stimulation with pD3/4 despite E5 possessing a higher avidity for peptide-MHC than C48 as demonstrated by staining with CD8-null tetramers. Cytokine production correlates better with TCR avidity than killing ability. Differences in avidity may affect the level of cytokine production to a greater extent than they do the killing – this is seen both between different clones (e.g. E5 vs C48) and between the different epitope interactions of a single clone (e.g. E5 stimulated with pD3/4 vs pD2). This could be of significance in dengue - it implies that despite similar lytic function high avidity cross-reactive T cells could produce higher levels of proinflamatory cytokines than serotype specific or partially cross-reactive T cells. It is important to remember that neither tetramer decay, nor the CD8-non-binding tetramers *directly* measure TCR affinity and the TCR/pMHC interaction is only one component, albeit perhaps the most significant, of a T cell's avidity for its target. Others include the TCR expression level, co-stimulatory molecule expression level and the extracellular microenvironment. A recent review has pointed out that T cells displaying all the characteristics of high avidity interactions may nonetheless bear a TCR that is of relatively low affinity (270). Evidence of this complexity is apparent in this study: whereas the vast majority of cross-reactive clones producing high levels of cytokines showed high avidity TCR/pMHC interactions independent of CD8, one (10H5) did not. All other cross-

reactive clones showed good or moderate binding to the CD8-null tetramer even though they did not produce cytokines as vigorously as E5 or 9F5.

A healthy debate continues regarding the relative importance of antibody and cellular immunity in the pathogenesis of severe dengue. This data suggests a middle road. Antibody enhancement of secondary infection facilitates viral infection of cells leading to high viraemia and antigen loading of antigen-presenting cells (114, 115). Memory CTL from a previous infection and happening to show cross-reactivity to this, the secondary viral serotype, are expanded. Many show high avidity for certain epitopes and these cells produce immunopathogenic levels of cytokines of both a type 1 and type 2 nature. Vasoactive cytokines contribute to plasma leakage. The large antigen load combined with the high avidity of the T cells results in over-activation, T cell exhaustion and cell death which may lead to clonal deletion. In the meantime a new pool of more serotype specific CTLs showing lower avidity has been generated. These survive into convalescence and have the potential to produce TNF-α, IFN-γ and GM-CSF in a therapeutic rather than immunopathogenic manner. Perhaps it is a lack of immunopathogenesis, as much as a degree of protection that is derived from these serotype-specific populations in patients with repeated exposure to dengue virus.

In conclusion, these findings have significant implications for understanding the role of virus-specific CD8+ T cells in immunity to dengue virus infection and in the pathogenesis of severe dengue disease. It will be important to assess the fine specificity, functional avidity and cytokine production of T cells elicited by putative dengue vaccine candidates in order to ensure protective immunity against all virus serotypes is elicited without leading to excessive pro-inflammatory cytokine release.

CHAPTER 5 CHARACTERISATION OF CD4+ T-CELL DENGUE-SPECIFIC CLONES SHOWING BROAD CROSS-REACTIVITY BETWEEN FLAVIVIRUSES.

Introduction

A number of CD4+ T cell dengue epitopes have been identified(187, 209-211, 271-276). CD4+ cells recognising such epitopes may do so in a serotype specific or cross-reactive manner (209-211) and this cross-reactivity may extend to the recognition of other flaviviruses. Much of the work that has characterised these responses has been from samples obtained from donors participating in experimental dengue vaccine trials. The NS3 protein is a rich source of both CD8+ and CD4+ T cell epitopes (205, 209, 210, 253, 273, 277) and this chapter describes the identification of an NS3 CD4+ epitope recognised by PBMC from a patient with natural infection, and the generation and characterisation of clones specific for it.

Materials and Methods

Preparation of NS3 peptide pools.

Overlapping 15 amino-acid peptides spanning the length of the NS3 protein (sequence given in appendix 1) of each dengue serotype were mixed into 47 pools each containing 23 peptides such that each peptide appeared in two different pools at a concentration of 83μM. Peptides were dissolved as described in chapter 2 and the construction of matrix is illustrated in Figure 48.

Pools	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
25	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
26	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
27	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
28	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
29	116	117	118	119	120	121	122	123	124														
30	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
31	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
32	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
33	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
34	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
35	116	117	118	119	120	121	122	123															
36	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
37	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
38	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
39	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
40	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
41	116	117	118	119	120	121	122	123	124														
42	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
43	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
44	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
45	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
46	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
47	116	117	118	119	120	121	122	123	124														

White Dengue 1 Dengue 2 Dengue 3 Dengue 4

Figure 48. The two-dimensional overlapping peptide matrix covering the whole of NS3 for each of the four dengue serotypes. Each peptide was represented in each pool at 83μM.

All pools were filtered through 0.22μm membranes – failing to filter resulted in false positives in control ELIspots using PBMC from flavivirus naïve individuals (Figure 49). This is likely to be due to contamination by particulate matter from poorly soluble peptides which has been shown to cause false positive ELIspot responses(278). Pools were used in an IFN-γ ELIspot assay at 8.3μM per peptide.

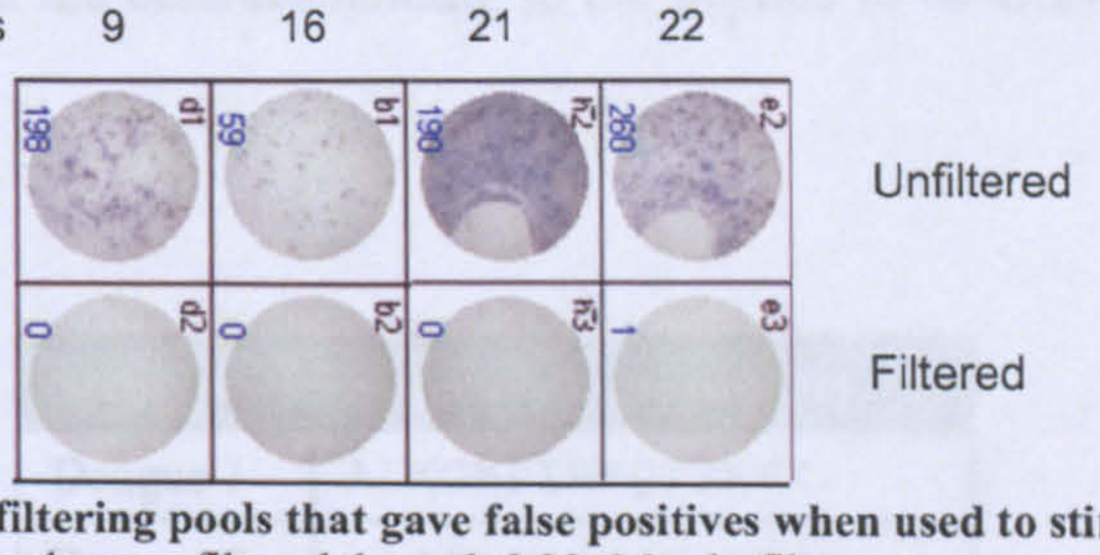


Figure 49. The effect of filtering pools that gave false positives when used to stimulate flavivirus naïve control patients. Pools were filtered through 0.22μM spin filters.

Results

Generation of CD4 positive clones showing cross-reactivity between all dengue serotypes.

IFN-γ ELIspots were performed using freshly thawed PBMC from 5 patients and all 47 NS3 peptide pools. Samples had been taken 2 weeks after acute illness. 2 of these demonstrated clear reproducible responses. One of these – BC429 – recognised a previously identified CD8 B*07 restricted epitope and is not discussed further here. The other patient – BC408 (DHF III, infected with DEN4) – showed a dominant response in all pools containing peptide 99. This differed between all 4 dengue serotypes (Figure 50) and its position within NS3 is illustrated in appendix 1. PBMC were stimulated with a pool of all 4 peptide 99 variants (each present at 10μM). On day 21 the resultant short term lines were screened in a cultured ELIspot to try and determine the restriction of the response. A number of different B-cell lines previously pulsed with peptide for 1 hour at 37°C at a concentration of 10 μM were used to present antigen. By serendipity the only two B cell lines that were recognised were the two positive for DRB1*15, an HLA molecule also expressed by patient BC408. Intracellular cytokine staining with surface staining for CD4 and CD8 after stimulation confirmed the cells responding to the peptide to be CD4 positive (Figure 51).

Serotype	Sequence of peptide 99
Dengue 1	ALKGMPIRYQTTAVK
Dengue 2	LRGLPIRYQTPARIA
Dengue 3	AMKGLPIRYQTTATK
Dengue 4	LRGLPIRYQTPAVKS

Figure 50. The sequence of peptide 99 for each of the four dengue serotypes.

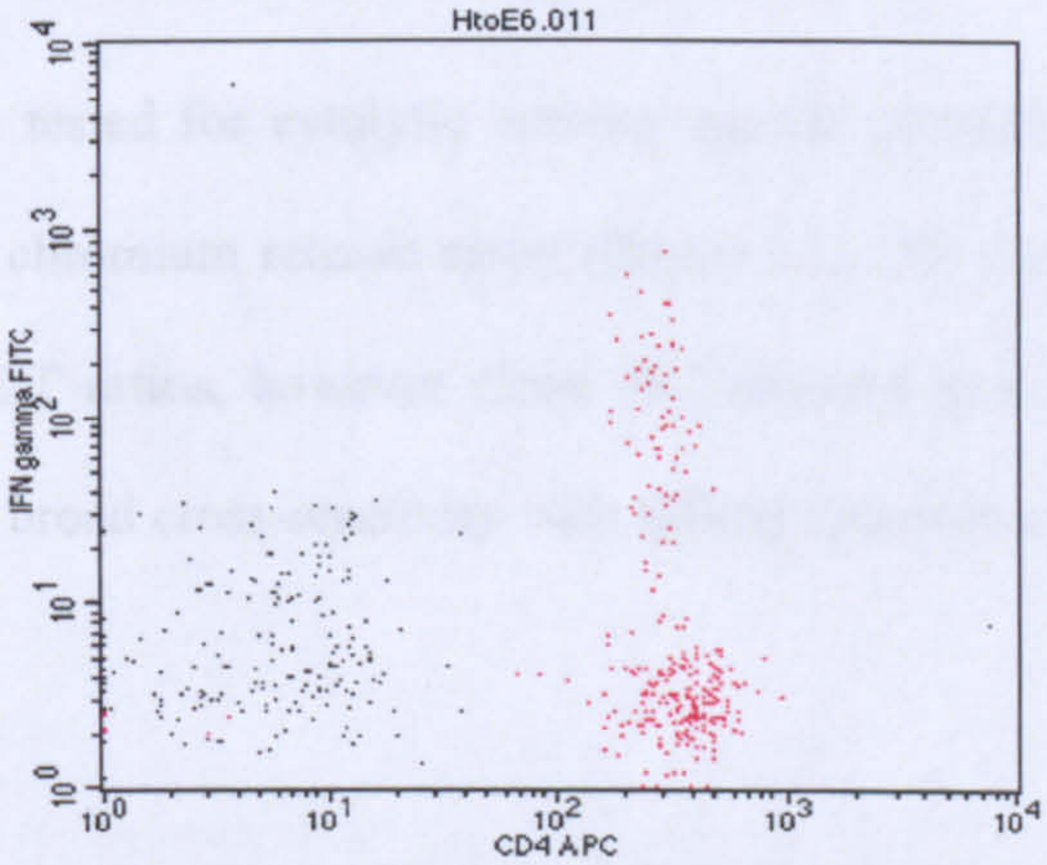


Figure 51. Cells within the short term line derived from BC408 recognising peptide 99 are CD4+. Intracellular IFN-γ staining of the short term line generated from BC408 after stimulation with peptide 99 pulsed DRB1*15+ B cells. Cells were incubated together for 4 hours before permeabilisation and staining with anti-IFN-γ and anti-CD4. The CD4+ cells are those producing IFN-γ.

Antigen specific cells were selected using the MACS IFN-γ capture kit as described in chapter 2. These cells were used for cloning by limiting dilution. Clones were tested for specificity at three weeks by cultured ELIspot in the presence of peptide-loaded HLA matched B cells. 5 clones showed dengue specific responses and were equally reactive against each of the 4 dengue variants. Clones recognising elements within the DEN3 variant of this 15mer have been described(211). The authors reported the generation of a CD4+ DEN3 specific clone that recognised the minimal epitope IRYQTTATK ($NS3_{241-249}$) as determined by killing assays. It failed to lyse cells pulsed with DEN1, 2, 4 or Yellow fever or West Nile Virus variants and lysis fell to around 5% with the loss of the initial isoleucine. Clones cross-reactive between all peptide serotype variants have however not previously been described.

BC408 dengue CD4+ clones show cytolytic activity

The 5 clones were tested for cytolytic activity against peptide pulsed DRB1*15 B cells in a standard chromium release assay (Figure 52). All showed some degree of cytolysis at high E:T ratios, however clone 4E3 showed particularly high activity. This clone showed broad cross-reactivity with killing apparent at E:T ratios as low as 1.25:1 (Figure 53).

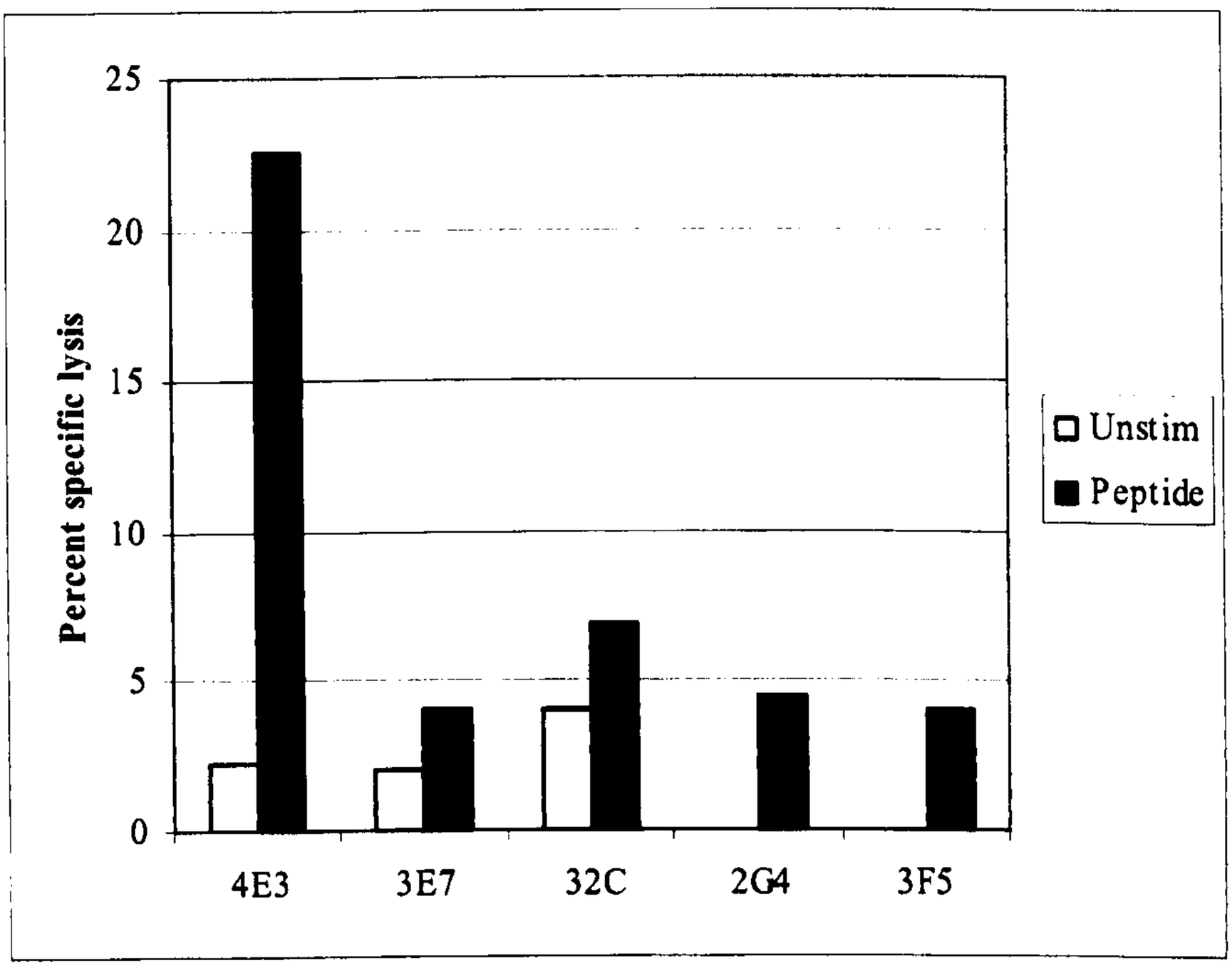


Figure 52. Chromium release assay using dengue specific clones derived from patient BC408. DRB1*15 positive B cells were pulsed with dengue 1 peptide 99 at 20μM for 1 hour 37°C and incubated with clones for 4 h ours at an E:T ratio of 20:1.

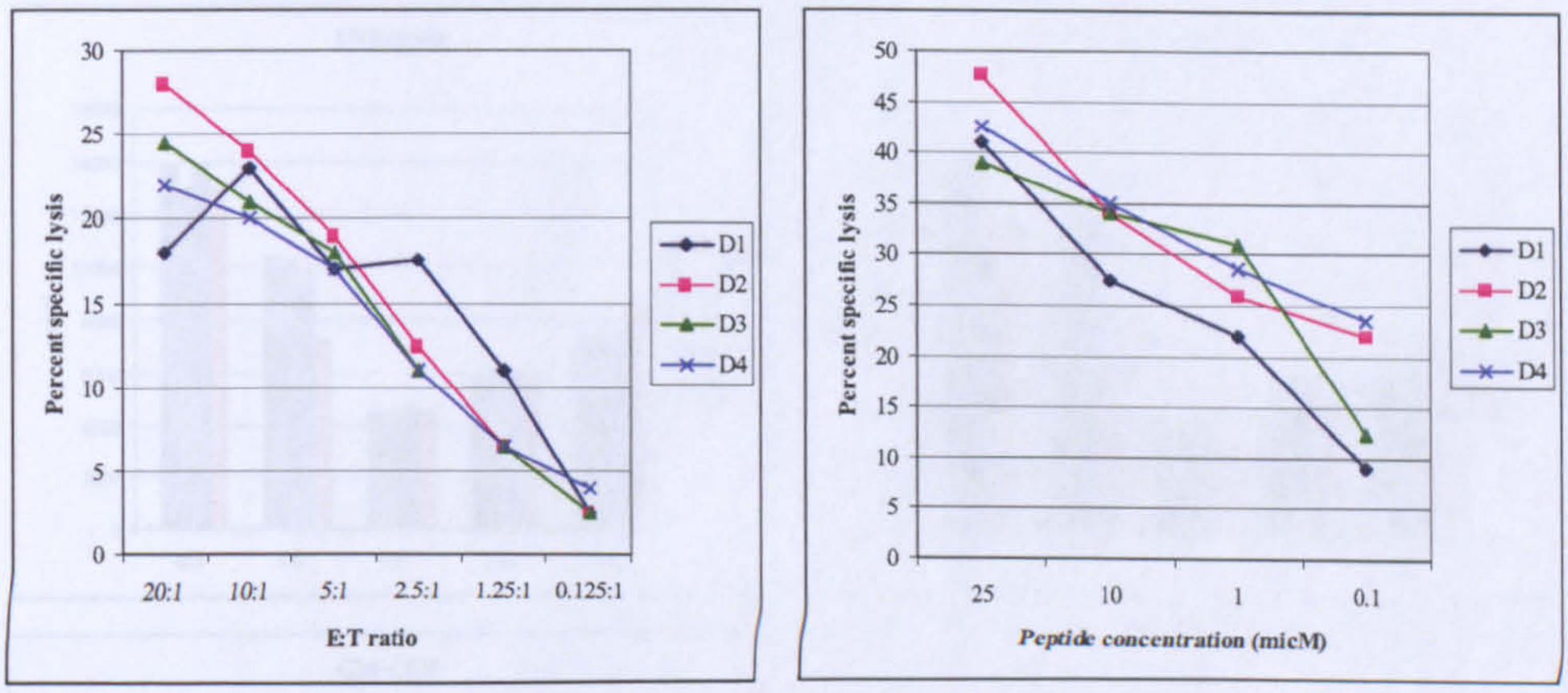


Figure 53. Clone 4E3 shows broad cross-reactivity across all 4 variants of peptide 99. Chromium release assays for clone 4E3 using DRB1*15 B cells pulsed with each of the four variants of peptide 99 for 1 hour at 37°C. **Left)** At different E:T ratios with target B cells being pulsed with peptide at 25μM. **Right)** At different peptide concentrations with an E:T ratio of 20:1.

Killing ability and cytokine production are correlated.

Clones were stimulated with matched B cells pulsed with 20μM of each dengue serotype variant of peptide 99. After 24 hours supernatant was removed and the cytokines produced measured (Figure 54). The cytokine profile of all clones tended to Th1 and those most effective at killing (e.g. 4E3 and 3F5) also produced the largest amounts of TNF-α, GM-CSF and IFN-γ for a given stimulation. Clones did produce IL-4 and IL-6 but at very low levels.

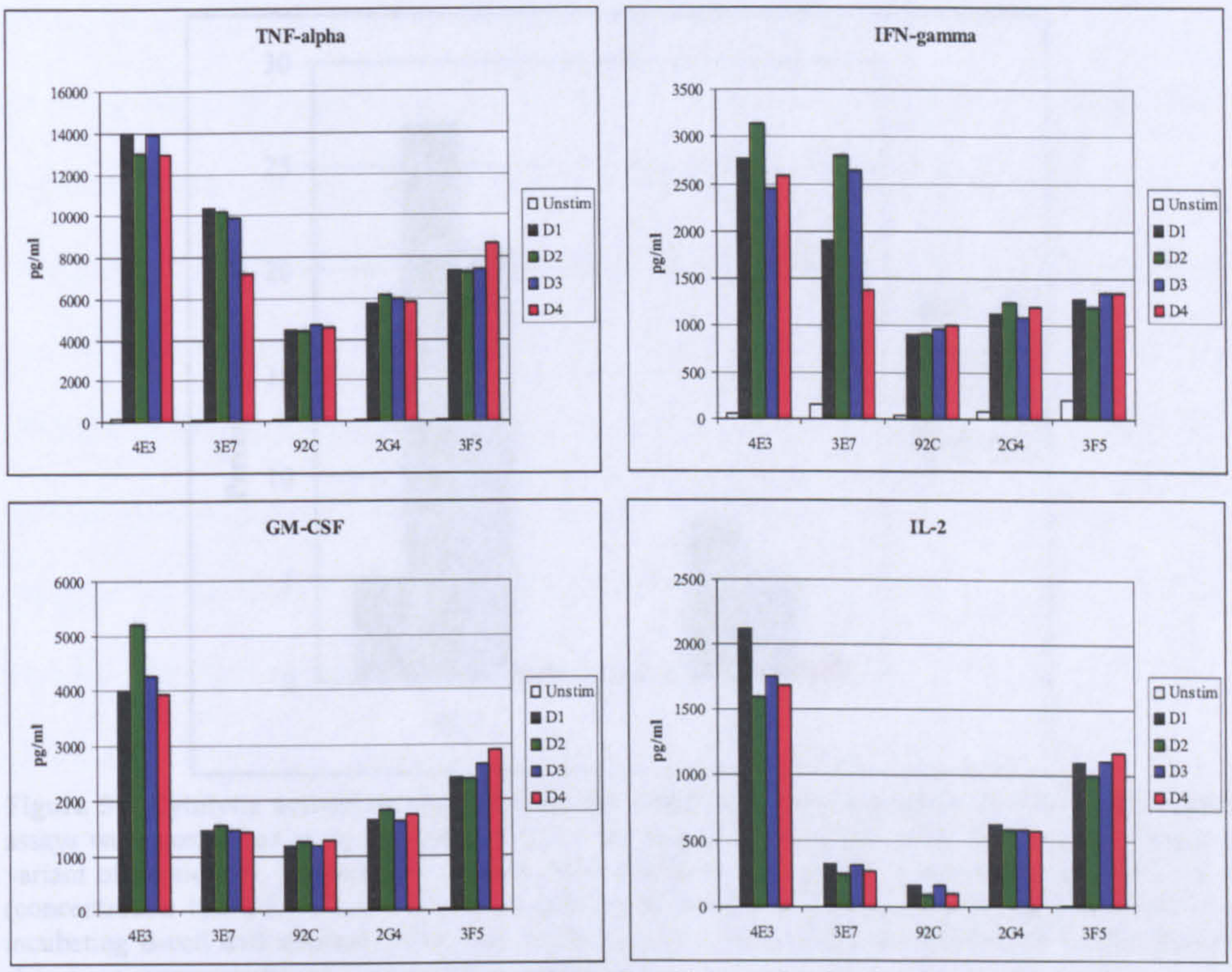


Figure 54. Clones produce Th1 type cytokines. Cytokine production by CD4+ clones derived from patient BC408 after stimulation with B cells pulsed with 20μM of each variant of peptide 99, or unpulsed B cells as a control. Cells were incubated at an E:T ratio of 20:1 at 37°C overnight. Tissue culture supernatants were removed and analysed on the Luminex system. This data is representative of 3 independent experiments.

BC408 cytolytic CD4+ clones recognising dengue kill almost entirely through perforin-mediated mechanisms.

Chromium release assays were carried out in the presence and absence of inhibitors of different killing mechanisms: an antibody against Fas and concanamycin A – an inhibitor of perforin mediated killing. Blocking Fas mediated mechanisms had little effect on killing whereas blocking perforin nearly completely abrogated killing by clone 4E3 and 3F5 (Figure 55).

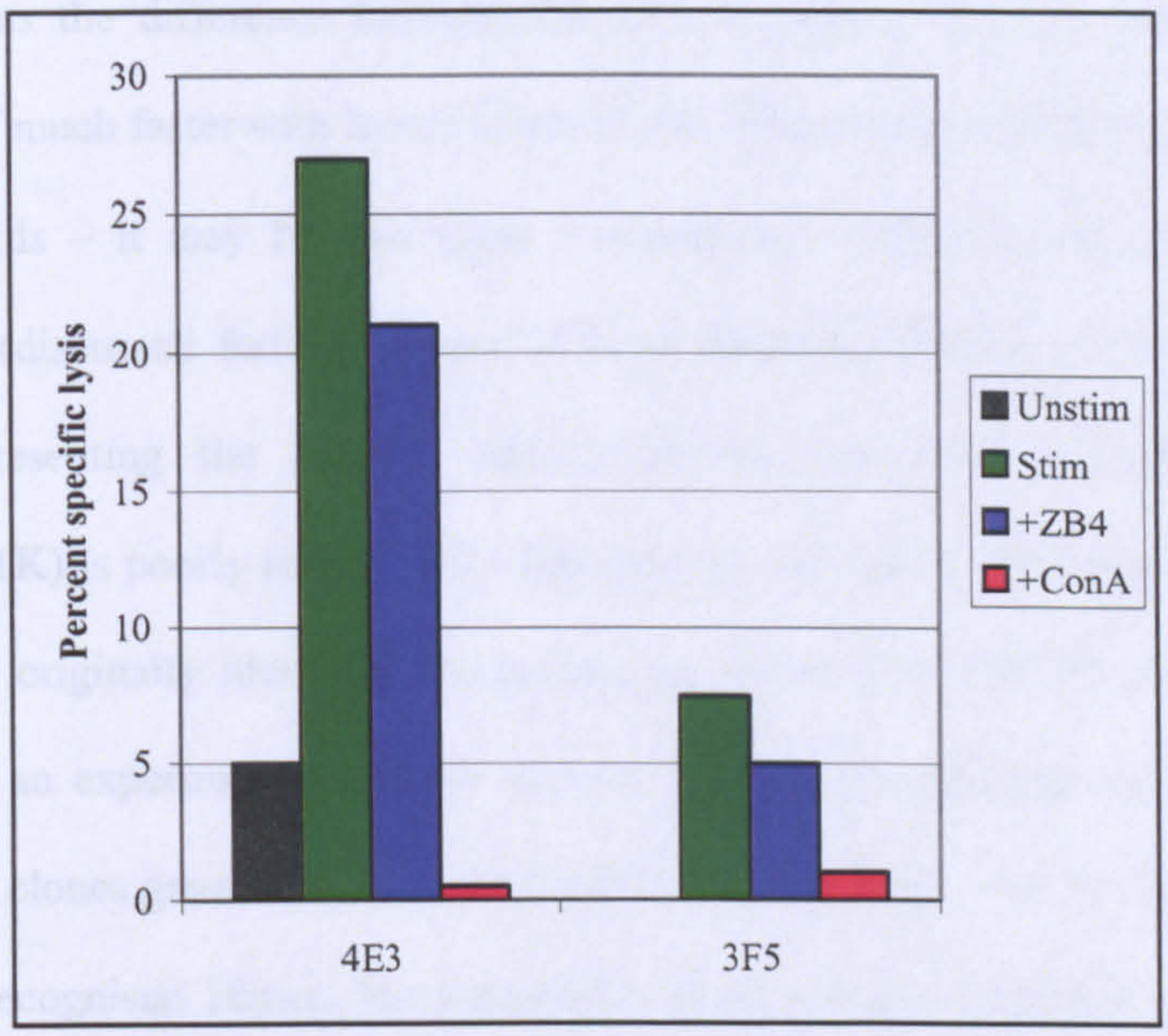


Figure 55. Cytolytic activity is abolished by the addition of concanamycin A. Chromium release assays were performed at an E:T ratio of 10:1 with targets being pulsed with 25μM of the dengue 1 variant of peptide 99. Perforin activity was inhibited by incubating the clones with concanamycin A (concentration 1μM) for 1 hour before the addition of B-cells. Fas-dependent killing was blocked by incubating B-cell with antibody ZB4 (5μL in 200μL) for 1 hour before the addition of T cells. Results shown are representative of 3 independent experiments.

Identification of the minimal epitope

The ability of clones 4E3 and 3F5 to recognise truncated peptides (Figure 56) was tested in a chromium release assay. Deletions from the distal end of the peptide representing DEN-1 produced modest falls in lysis, whereas deletions from the proximal end produced significant falls. Interestingly recognition of peptide 5L was better than the preceding 4L, presumably because the methionine in 4L is a less favourable terminal amino acid than the proline in 5L. Once this proline is removed recognition halves. 5L and 5R precipitate similar levels of lytic activity from clone 4E3. 3F5 however recognises 5R less efficiently than 5L. These peptides were titrated in a cultured ELIspot assay (Figure 57). From similar levels of activity at high peptide

concentrations the difference between the two is quickly revealed with responses dropping off much faster with lower levels of 5R. These two peptides overlap by only 5 amino acids – it may be that these 5 represent a critical region necessary for recognition (discussed further below). It is of particular interest that the truncated variant representing the DEN-1 version of the previously described epitope (IRYQTTATK) is poorly recognised – lysis drops to around a third of maximal. This epitope was originally identified by generating clones from PBMC taken from the recipient of an experimental DEN-3 vaccine – clones recognising only the DEN-3 variant. The clones generated here recognised all four variants with 5L being the most efficiently recognised 10mer. No truncated peptide was as efficiently recognised as the 15mer.

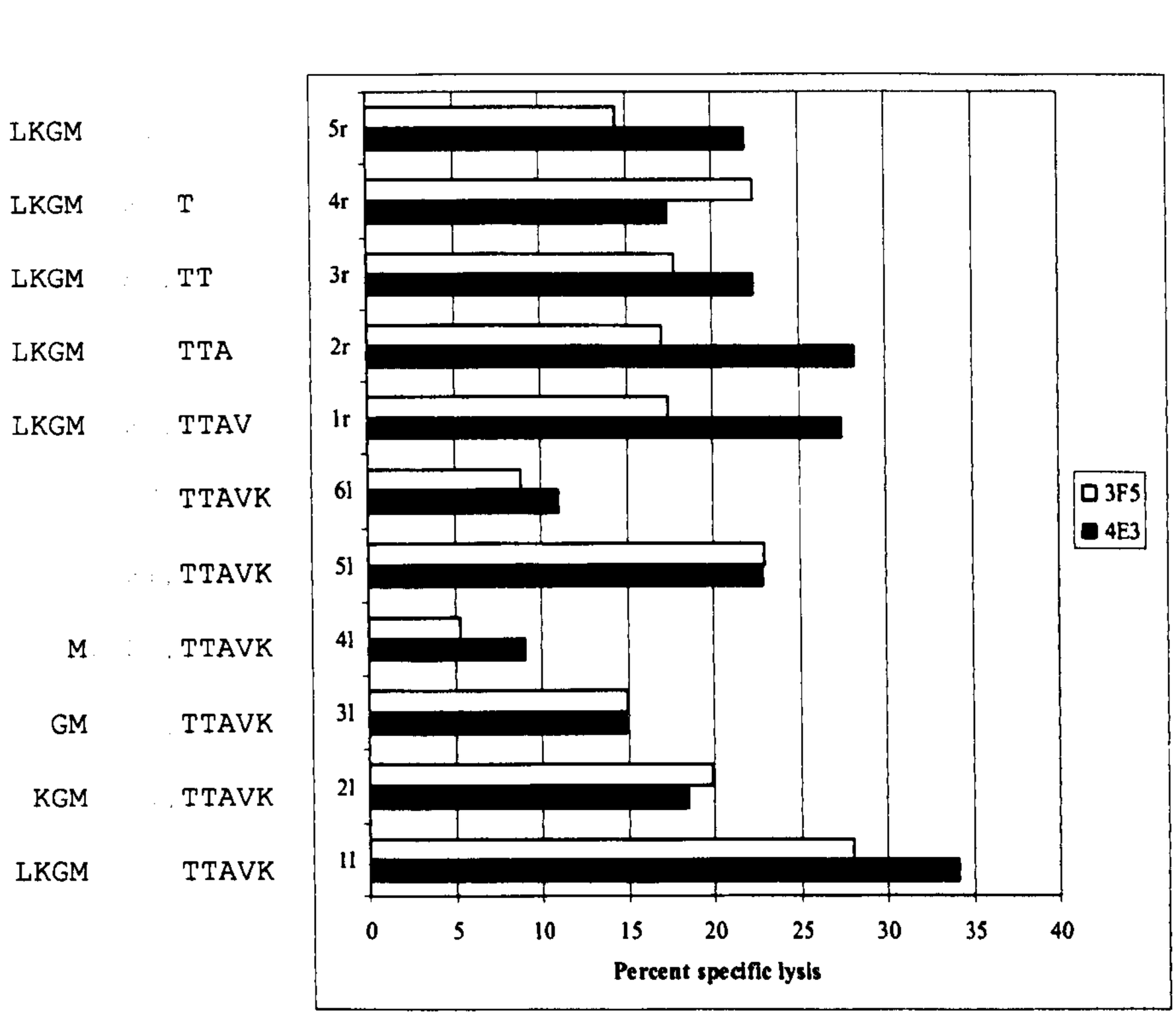


Figure 56. The variation in lysis efficacy with truncated versions of dengue 1 peptide 99. Clones were incubated for 4 hours with B cells pulsed with the indicated peptide at an E:T raito of 10:1.

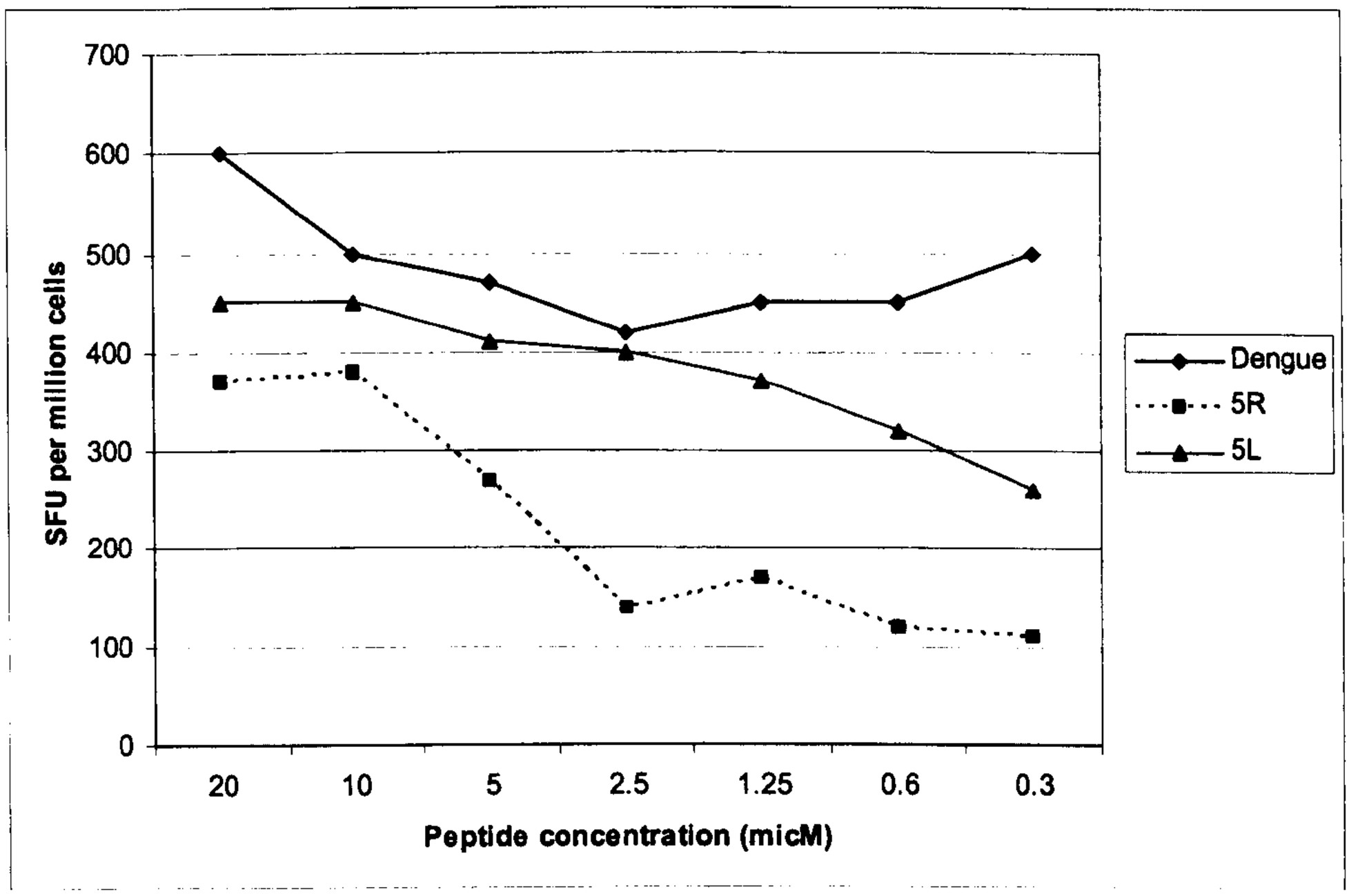


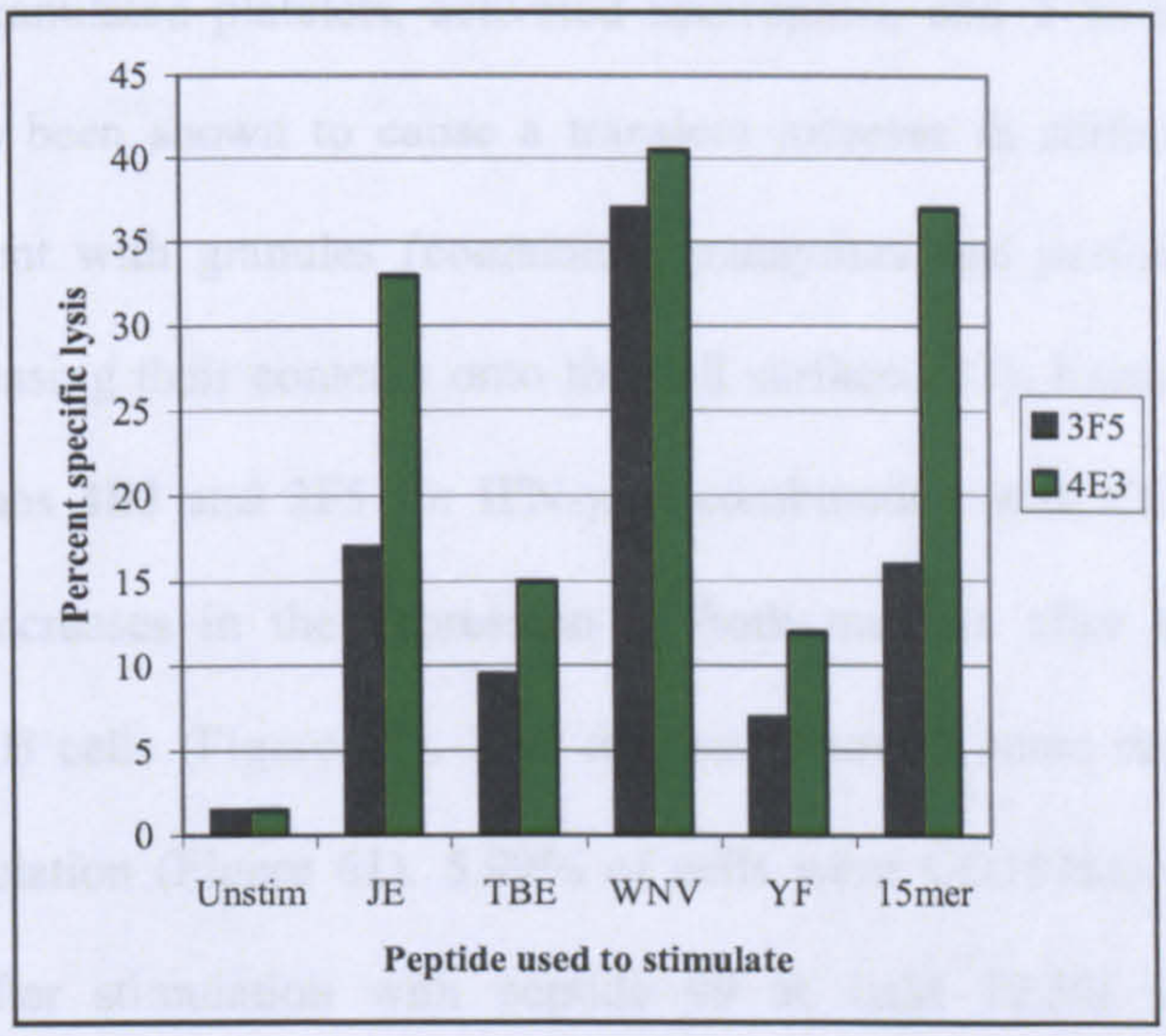
Figure 57. 5L shows a more sustained response in peptide titrations than 5L. Clone 4E3 was stimulated with B-cells pulsed with a titration of peptide in a cultured ELIspot. The response to dengue wild-type peptide 99 was sustained throughout. The response to 5L dropped off at low concentrations but not as strikingly as 5R.

Clones show broad cross-reactivity between flavivirus epitopes despite considerable sequence variation.

Clones 4E3 and 3F5 were tested in chromium release assays against targets pulsed with peptides representing sequences equivalent to the dengue epitope from the NS3 of different flaviviruses. Despite considerable differences (Figure 58) the clones were able to effectively kill most variants (Figure 59 – top panel) with equal efficacy against B cells presenting the West Nile Virus variant peptide and decreased efficacy against those with the greatest variation from the dengue sequence. Peptide titration in a cultured ELIspot assay demonstrated that despite similar lytic activity at higher peptide concentrations the response to stimulation with the Japanese encephalitis variant dropped off very quickly at lower concentrations (Figure 59 – bottom panel).

Flavivirus	Peptide Sequence
Japanese encephalitis	--R-L-V----S-Q-
Tick borne encephalitis	--N-KRV-FHSP-G-
West Nile Virus	--R-L------S---
Yellow fever	-FH-LDVKFH-Q-FS
Dengue 15mer	ALKGMPIRYQTTAVK

Figure 58. Variations in the sequence of dengue 1 peptide 99 between other flaviviruses. "-" indicates homology with the dengue reference sequence.



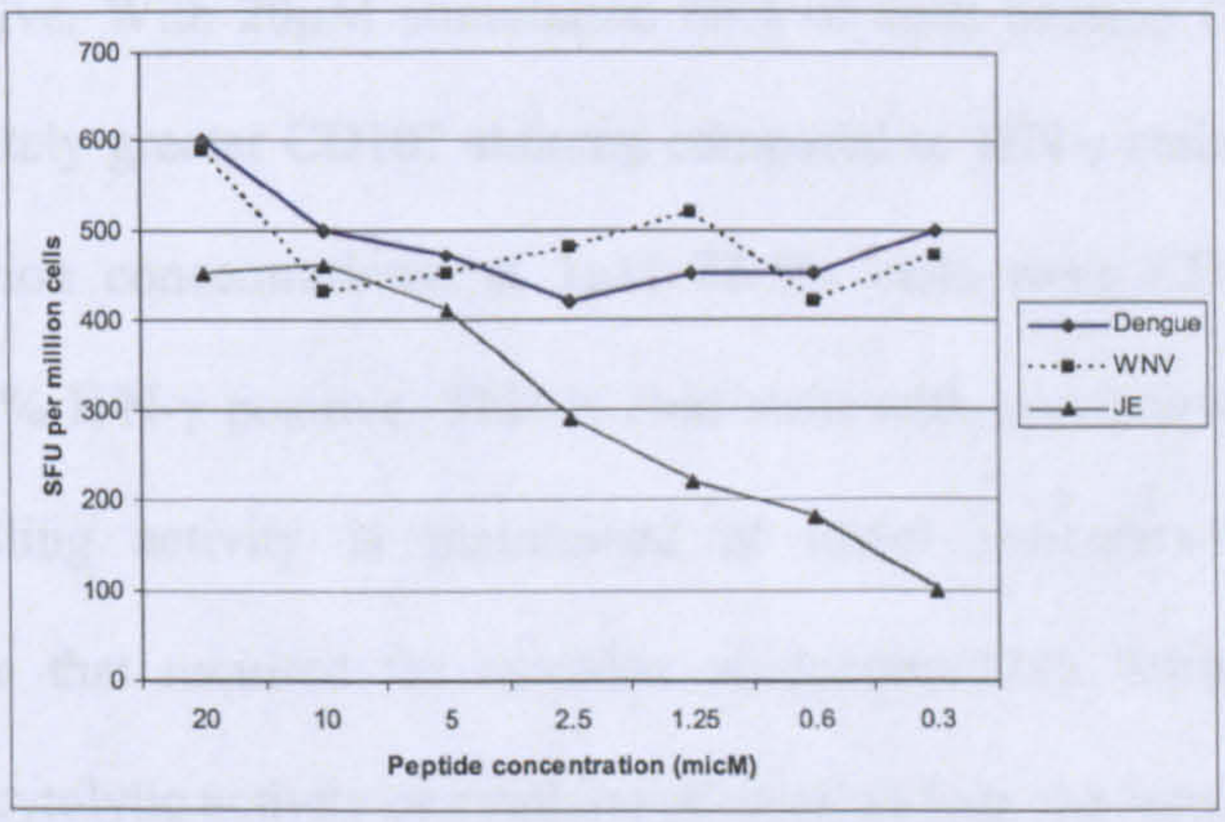


Figure 59. Clones 4E3 and 3F5 show broad cross-reactivity across flavivirus variants of the dengue 1 peptide 99. JE: Japanese encephalitis, TBE: Tick borne encephalitis, WNV: West Nile virus, YF: yellow fever, 15mer: dengue 1 peptide 99, Unstim: B cells with no peptide pulse. **Top)** Clones were incubated for 4 hours with peptide pulsed chromium labelled B cells at an E:T ratio of 10:1 in a standard CTL lysis assay. **Bottom)** Peptide titration in a cultured ELIspot assay demonstrates the rapid fall in response with JE variant stimulation despite similar levels of activity at high concentrations.

CD107a/b expression is a marker of CD4+ cell degranulation

CD107 is a marker of degranulation and has been demonstrated to be a surrogate of CTL lytic activity. Originally known as lysosome-associated protein it exists in two forms: LAMP-1 (CD107a) and LAMP-2 (CD107b) – both are membrane glycolipids that provide selectins with carbohydrate ligands. They are found in the membranes of lysosomes, degranulated platelets, activated neutrophils, and T cells. Activation of CD8+ cells has been shown to cause a transient increase in surface expression of CD107 consistent with granules (containing granzymes and perforin among other substances) releasing their contents onto the cell surface(237). Intracellular cytokine staining of clones 4E3 and 3F5 for IFN-γ in combination with CD107a/b staining demonstrated increases in the expression of both markers after stimulation with peptide pulsed B cells (Figure 60). This response showed some relationship to the degree of stimulation (Figure 61). 5.99% of cells were CD107a/b positive without stimulation. After stimulation with peptide 99 at 1μM 72.5% of cells became CD107a/b positive. With 20μM stimulation 88% of cells became CD107a/b. There was proportionately greater CD107 staining compared to IFN-γ staining, particularly at low stimulation concentrations: at 1μM 72.5% cells were CD107a/b positive, compared to 45% IFN-γ positive. This is consistent with previous observations that lymphocyte killing activity is maintained at lower concentrations of peptide stimulation than that required for cytokine production(279). Stimuli that induced lower levels of cytolytic activity or cytokine production (e.g. the Japanese encephalitis variant of NS3 p99) resulted in similarly low levels of CD107a/b and IFN-γ expression. Despite its effect on killing activity (Figure 55), the addition of concanamycin A during incubation did not reduce the CD107a/b or IFN-γ response (Figure 62). In fact for reasons that are not clear the CD107a/b expression increased

slightly (this phenomenon, albeit less pronounced, was also noted with ConA treated CD8+ clones – see figure 46 in chapter 4). Colchicine (an inhibitor of microtubule activity as a whole) did reduce CD107a/b expression. This is consistent with concanamycin A's mode of action: it is thought to block perforin mediated killing mainly through acidification of endosomes causing increased perforin degradation. Thus granules can still be transported to the surface (increasing levels of surface CD107) but their contents are ineffective.

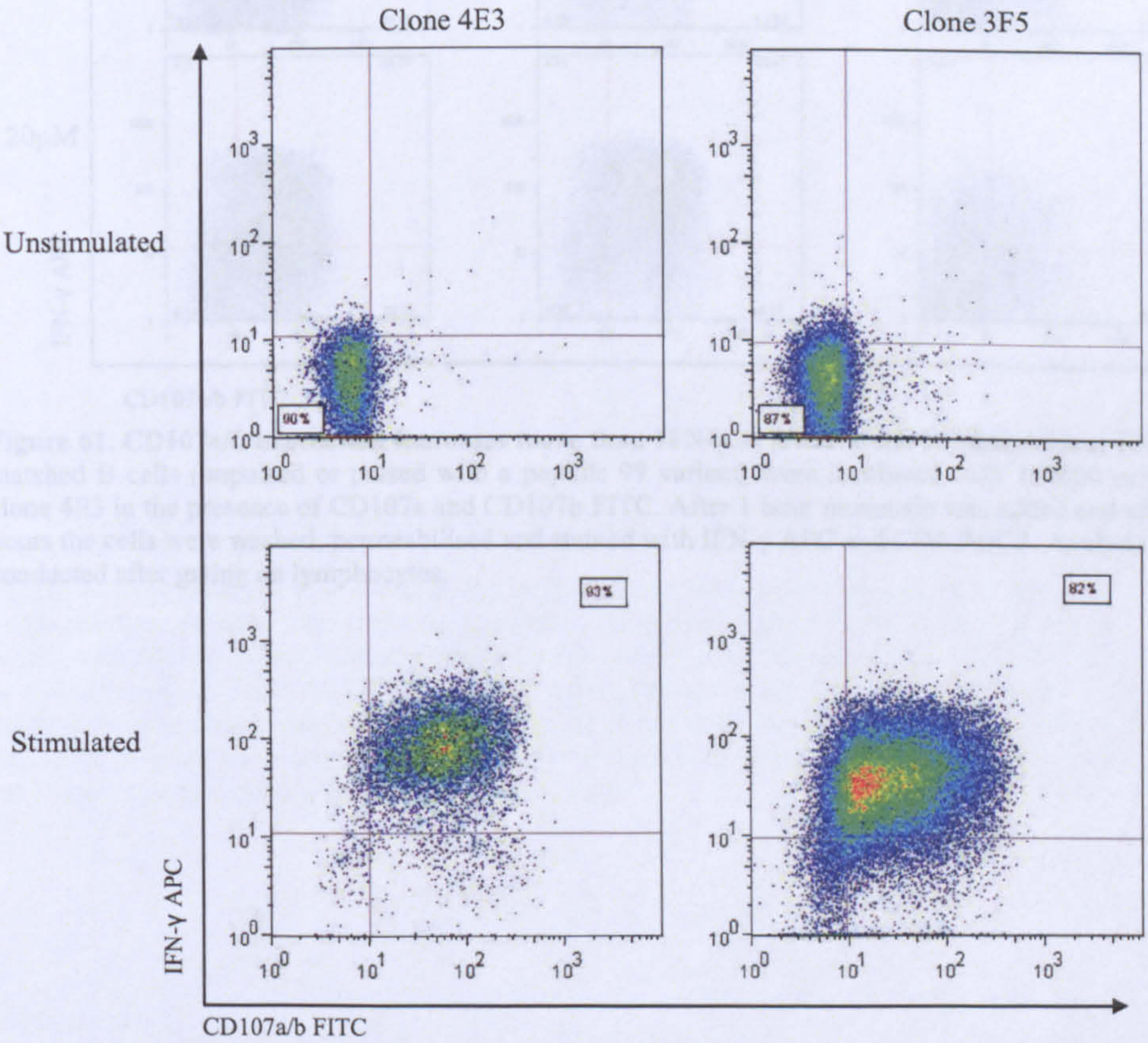


Figure 60. All BC408 derived CD4+ clones increased CD107a/b surface expression following antigen stimulation regardless of the effectiveness of their cytolytic acitivity. 100000 matched B cells (unpulsed or previously pulsed with 20μM dengue 1 peptide 99) were incubated with 100000 cells of clone 4E3 and 3F5 in the presence of CD107a and CD107b FITC. After 1 hour monensin was added and after 5 hours the cells were washed, permeabilised and stained with IFN-γ APC and CD4 PerCP. Analysis was conducted after gating on lymphocytes. 4E3: 90% of cells fall below threshold for IFN-γ and CD107 staining before stimulation. After stimulation 93% of cells fall above. Similar changes were apparent with 3F5 and the other clones derived from patient BC408.

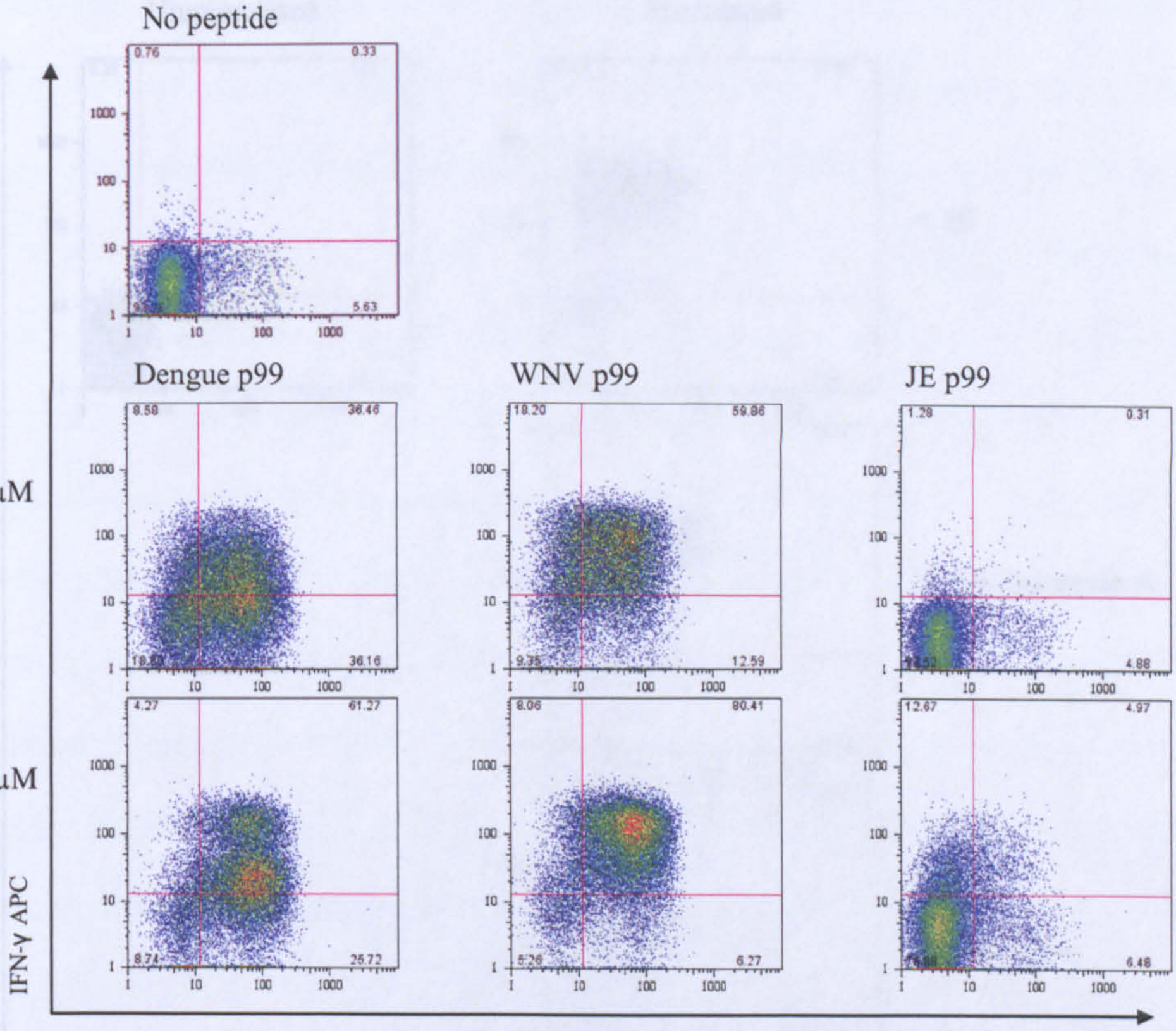


Figure 61. CD107a/b expression increases more than IFN-γ at lower levels of stimulation. 100000 matched B cells (unpulsed or pulsed with a peptide 99 variant) were incubated with 100000 cells of clone 4E3 in the presence of CD107a and CD107b FITC. After 1 hour monensin was added and after 5 hours the cells were washed, permeabilised and stained with IFN-γ APC and CD4 PerCP. Analysis was conducted after gating on lymphocytes.

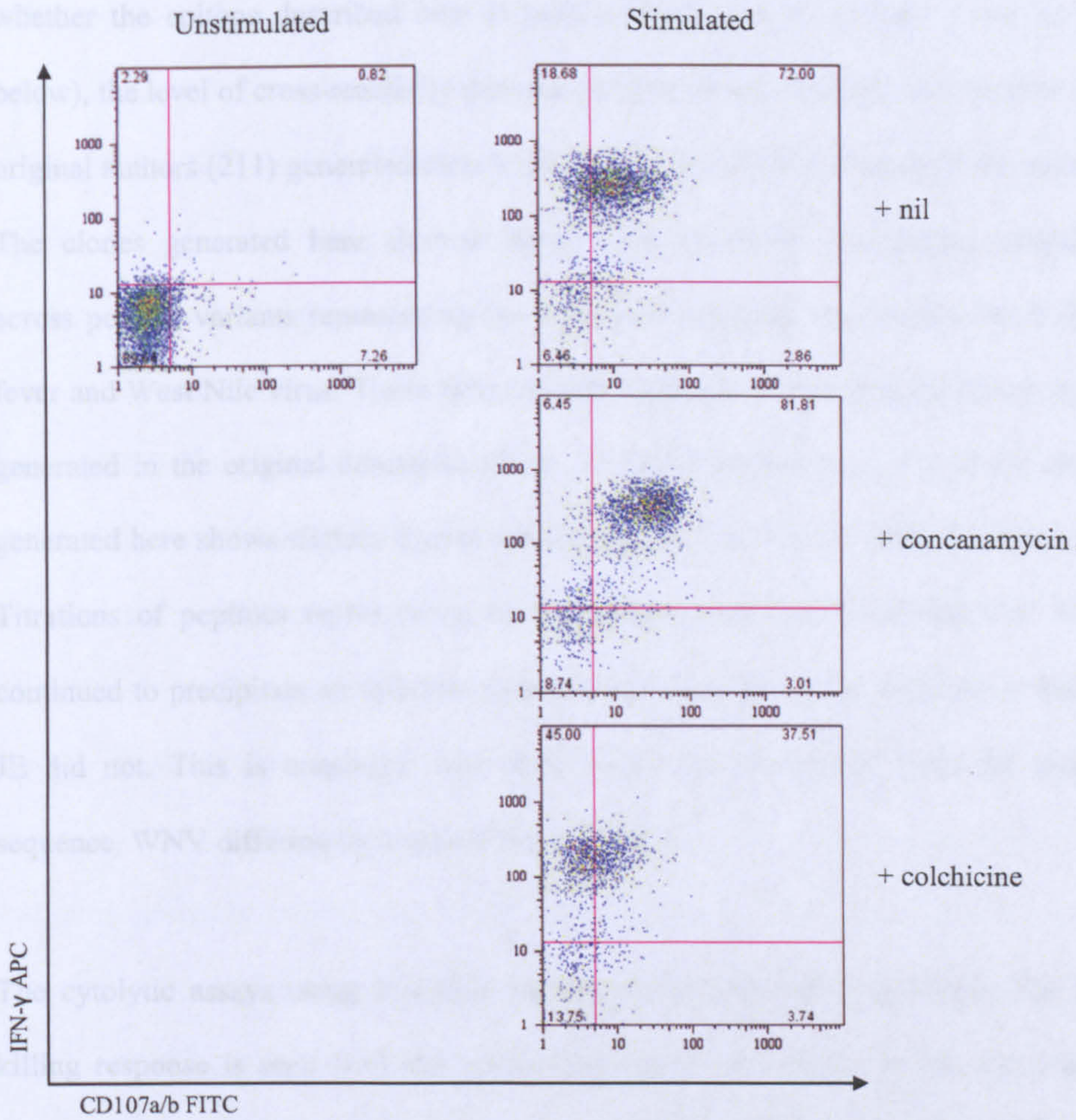


Figure 62. The addition of concanamycin A to clone 4E3 eliminates cytolysis but does not reduce degranulation marker CD107a/b upregulation. 100000 matched B cells (unpulsed or previously pulsed with 20μM dengue 1 peptide 99) were incubated with 100000 cells of clone 4E3 in the presence of CD107a and CD107b FITC. After 1 hour monensin was added and after 5 hours the cells were washed, permeabilised and stained with IFN-γ APC and CD4 PerCP. Analysis was conducted after gating on lymphocytes. Concanamycin A (1μM) or colchicine (20μM) were added to the T cell clone for 1 hour prior to exposure to B cells in certain cases.

Discussion

It is generally accepted that fluid leak in dengue is largely due to the release of vasoactive cytokines. CD4+ cells are important sources of these cytokines – their depletion in experimental systems abrogates the dengue-specific cytokine response(213) – and also play a key role in priming naïve CTLs. Regardless of

whether the epitope described here is novel (which can be debated - and is. See below), the level of cross-reactivity demonstrated by clones recognizing it is new. The original authors (211) generated clones specific for the DEN-3 version of the epitope. The clones generated here showed broad cross-reactivity, recognition extending across peptide variants representing the 4 dengue serotypes to include even Yellow fever and West Nile virus. These heterologous viruses were not recognized by clones generated in the original description(211). At low concentrations one of the clones generated here shows slightly higher activity against DEN-2 and DEN-4 (Figure 53). Titrations of peptides representing the heterologous viruses confirmed that WNV continued to precipitate an effective cytolytic response at low concentration whereas JE did not. This is consistent with their respective divergence from the dengue sequence, WNV differing by 3 amino acids, JE by 5.

The cytolytic assays using truncated peptides raise interesting questions. The best killing response is seen with the 15mer, yet significant activity is preserved using certain peptides truncated from either end. It is fascinating that peptides 5R and 5L, both of which demonstrate a relatively well preserved ability to precipitate lysis have only 5 amino acids in common, PIRYQ. How could this be sufficient for TCR recognition when class II epitopes are "supposed" to be at least 13 amino acids long(100)? It implies that although the 15mer is optimal, significantly shorter peptides can contain a critical, necessary portion of the epitope. Studies of the structure of the TCR interaction with peptide-MHCII demonstrate that although class II epitopes are classically longer than class I epitopes (the "sausage" that sticks out each end of the "hotdog") the TCR interaction is restricted to a 9 amino acid portion of the whole(280). Most MHC class II molecules have four binding pockets occupied by

amino acids 1, 4, 6, and 9 of the minimal peptide epitope, while the residues at positions 2, 3, 5, 7, and 8 are available to interact with the TCR(281). Thus the remainder of MHC class II bound peptides (the "flanking regions") are situated outside of this core. So while MHC class II molecules capture peptides of substantially larger length, only a subset of residues is "read out" by the bound TCR. The P5 residue of the MHC-bound peptide appears to play a critical role in TCR binding suggesting that mutations affecting non-P5 positions may be less detrimental to the recognition process. This might explain why these 2 truncated peptides with only 5 amino acids in common are still able to produce a significant response – the PIRYQ portion may represent a critical motif necessary for binding. For optimal activity however more is required, and it appears that the regions flanking the "peptide core" contribute to this more optimal recognition. This is thought to be mediated primarily by the creation of a more stable interaction with the class II molecule rather than directly playing a part in binding to the TCR(281). Others have made similar observations(282): In the process of characterizing an epitope recognized by CD4+ cells from an HIV positive patient who had received an experimental HIV vaccine, Ondondo *et al found that 2 overlapping 16*mers sharing a common core of just 9 amino acids were both recognized by CD4+ cells (RDYVDRFFKTLRAEQA and FKTLRAEQATQEVKNW). Recognition was lost if even a single amino acid from this common core was removed from the appropriate end. A comparable phenomenon is seen with the loss of proline between truncated peptide 5L and 6L (figure 56). Returning to the original epitope description by Kurane et al(210) armed with these insights it worth noting that their minimal epitope stops short of containing the entire PIRYQ sequence. They defined their minimal epitope as IRYQTTATK on the basis that lysis dropped to 5% with truncated peptide

RYATTATK. Our clones produced around 23% lysis of B cells loaded with PIRYQTTAVK, dropping to 5% with the loss of the proline (IRYQTTAVK). It is therefore possible that these two minimal peptides represent two distinct epitopes which although presented by the same HLA molecule are recognized by the T cell in distinct manners.

These observations go some way to explaining the high degree of cross-reactivity demonstrated by these clones. The West Nile virus variant of the dengue epitope is recognized by the two clones tested as least as well as dengue itself (figure 59) despite 3 amino acid differences. The PIRYQ motif remains intact. The Japanese encephalitis variant is much less well recognized, perhaps as a consequence of a substitution within this motif. The other variants tested have still more differences and are poorly recognized. It has been observed that CD4+ T cells show greater tolerance of changes in an epitope than do CD8+ T cells, and in fact demonstrate greater degeneracy overall(258): a single TCR is capable interacting with several class II molecules as well as recognizing class II epitopes with no sequence or physical homology at all(283). This promiscuity is thought to be a consequence of the positional flexibility the "open-groove" of a class II molecule allows its long epitopes. One could be mistaken for thinking that cross-reactivity was a deleterious phenomenon, lying behind immunopathology of all kinds. But of course such degeneracy is extremely beneficial. It means that an individual's limited array of TCRs is capable of recognizing a much greater repertoire of epitopes than would otherwise be possible(181). CD4+ T cell cross-reactivity may play an important part in both protection from and the pathology of secondary dengue. Cytokine production is the main means of CD4+ T cell effector action(284) and it could be hypothesized that the

promiscuous nature of CD4+ helper cells would allow the rapid expansion of cross-reactive populations from memory early in secondary infection. These could serve both as factories for vasoactive cytokines in their own right, as well as providing help to CD8+ T cells and other immune system components. The fact that dengue T cell clones are cross-reactive with other flaviviruses raises once again the question as to whether prior infection with another flavivirus might produce an anamnesiatic immune response to a primary dengue infection. Such a question will probably never be answered in the laboratory – large epidemiological investigations would be required.

These clones showed a Th1 phenotype, producing high levels of TNF-α and IFN-γ. The amount of IFN-γ produced is similar to that produced by the CD8+ clones described in chapter 4, whereas the amount of TNF-α produced by the CD4+ clones is an order of magnitude higher. This confirms CD4+ cells as important producers of cytokines and they are likely to be significant contributors to the high concentration of certain (particular vasoactive) cytokines associated with severe disease. The amount of TNF-α produced by each clone tested did not differ greatly when stimulated by peptides representing each of the four dengue serotypes. It has been theorized that a bias to TNF-α production over IFN-γ production might be associated with severe disease: Mangada et al(215) noted that the ratio of TNF-α to IFN-γ producing CD4+ T cells was higher after stimulation with inactivated dengue antigens from viral serotypes heterologous to that used in the vaccine preparation than with homologous antigen.

The cytolytic ability of certain of the CD4+ clones is of uncertain significance. The original observations relating to cytolytic CD4+ T cells were made in cell lines and *in vitro* clonal work such as that contained in this thesis – skeptics argued that such cells were an *in vitro* phenomenon with no physiological significance(285). In recent years however cytotoxic CD4+ T cells have been detected *in vivo* in association with viral infections such as CMV, HIV(286) and EBV, as well as autoimmune conditions (e.g. rheumatoid arthritis). *Ex vivo* phenotypic analysis suggests they represent antigen-experienced cells (memory) at an advanced stage of cellular differentiation(285) (CD27-, CD28-, CD57+) which generally kill by perforin mediated mechanisms - perforin is expressed constitutively in memory CD8+ T cells but is dependent upon cell activation in memory CD4+ T cells(287). As such it is likely that cytotoxic CD4+ T cells are of most importance in chronic disease (e.g. autoimmune) and persistent viral infection (up to 50% of CD4+ T cells in some HIV infected donors exhibit cytotoxic potential) with immune activation a key driving force behind their differentiation. This would help explain why they were first observed in experimental *in vitro* conditions. It is unlikely that such cytotoxic activity is of great significance in an acute viral infection although in theory memory populations might become differentiated enough during secondary infection to develop cytotoxic potential.

Clone 4E3 demonstrated considerable perforin mediated cytolytic activity. This lytic activity was paralleled by an increase in surface CD107a/b expression. CD107 has been used as a surrogate marker for the cytolytic activity of CD8+ CTL(237, 288) and NK cells(289) but has not been widely used in the assessment of CD4+ cytolytic activity. Peptide stimulation of these cytolytic CD4+ T cell clones also increased intracellular cytokine staining for IFN-γ. Consistent with the data from chromium

release assays cytokine staining fell more markedly than did CD107 staining as the peptide concentration used to stimulate the clones was reduced. Strictly speaking CD107 is a marker of degranulation rather than cytolysis *per se*. Rendering the contents of those granules ineffective with an agent such as concanamycin A eliminates true cytolytic activity (as assessed by chromium release assays) but does not decrease CD107 expression. In fact a slight increase was observed. The reason for this is not apparent.

It would be interesting to assess CD107 staining in CD4+ populations in fresh blood samples during acute dengue. Betts et al(237) found that directly staining CD8+ T cells from HIV patients with CD107 antibody *ex vivo* gave poor results. Staining after a short period of antigen specific stimulation produced clear populations of CD107+ CD8+ T cells which were also IFN-γ positive by intracellular cytokine staining. Direct *ex vivo* staining of PBMC from acute dengue patients with CD107 antibody gave poor results with no significant staining of either CD4+ or CD8+ T cells (data not shown). Prior stimulation of PBMC with either individual peptide epitopes or pooled overlapping peptides covering NS3 was also disappointing. Perhaps using fresh rather than frozen samples, or stimulating cells with a more "physiological" and effective antigen (e.g. inactivated viral lysate) would produce better results although of course it is possible (even likely) that CD4+ cells in acute dengue demonstrate no cytolytic activity either *in vivo* or *ex vivo*.

In conclusion this work confirms CD4+ cells as an important source of inflammatory cytokines. An inherent tendency to degeneracy (cross-reactivity) render these cell populations particularly likely to recognize heterologous viral epitopes (from either

dengue or other flaviviruses) and they could well play an important part in secondary infection, for good or for ill. Others have shown subtle differences in the balance of cytokines produced by dengue specific CD4+ cells undergoing heterologous stimulation which may affect disease severity. CD4+ clones derived from different individuals display different levels of cross-reactivity to a given epitope – presumably as a result of host and environmental factors (e.g. an individual's infection history, their other HLA types). The presence of cross-reactive memory CD4+ cells may render an individual more likely to develop severe clinical disease when infected with a heterologous dengue virus, particularly if the viral load is very high following antibody dependent enhancement.

CHAPTER 6 SUMMARY AND CONCLUSION

Dengue is a growing public health problem and – with the imminently possible exception of avian flu – perhaps one of the most significant *emerging* diseases in the world today(5, 6). Epidemiological observations made in the 1970s demonstrated that severe forms of dengue were seen most frequently in those experiencing secondary infection(7). It was proposed that this phenomenon could be accounted for by a process of antibody dependent enhancement(8, 120, 128). This association of severe disease with previous infection has been a stumbling block to vaccine development due to legitimate fears of immunisation-mediated disease enhancement(226).

Given these complications it is all the more important to have a good understanding of the immunopathogenesis of dengue disease. ADE accounts for many clinical and epidemiological observations. However there are features it cannot explain: not all patients experiencing secondary infection develop DHF(28, 135) and not all cases of severe disease are seen solely in secondary infection. ADE provides a convincing hypothesis of the mechanism lying behind the increased viral load noted in some severe disease but does not in and of itself provide sufficient explanation for all the pathological features of DHF. As the need for a safe and effective vaccine becomes ever greater it is important to develop a more holistic understanding of dengue pathology.

This thesis builds on a body of evidence that has accumulated in recent years that supports a role for cellular mediated immunity in the pathogenesis of severe dengue fever. CTL have been shown to mediate immunopathology in secondary heterologous

LCMV infection(188) through "original antigenic sin"-like mechanisms. A similar phenomenon has been described in dengue patients(189) and mouse work with other viruses has shown that heterologous immunity to a virus *unrelated* to the infecting virus is capable of mediating immunopathology(192). In addition CD4+ T cells are important producers of cytokines in dengue infection(213) and are capable of demonstrating broad cross-reactivity across dengue serotypes and even other flaviviruses. The T cell memory pool is not a static cell repository but is rather modulated by each infection it encounters, sometimes in a manner capable of contributing to disease severity. Such a phenomenon is highly likely to be of significance in the immune response to dengue virus infection, with its four relatively stable heterologous serotypes. In the following section the key findings of this thesis are discussed along with directions for future work.

A subset of high avidity dengue serotype cross-reactive CTL with greater cytolytic and cytokine producing activity than serotype-specific cells were present in acute disease but absent in convalescence.

Cross-reactive CD8+ T cells could be expanded in short term culture from patients with acute dengue but could not be detected or expanded from convalescent samples taken from the same patients. Dengue specific CD8+ clones recognising the A11 GTS epitope were generated from dengue patients. Cross-reactive clones recognising at least two dengue serotype epitope variants were, with few exceptions, of higher avidity than serotype-specific clones. These 2 populations differed in their cytolytic activity and cytokine production. Highly avid cross-reactive clones produced higher levels of both type 1 and type 2 cytokines (many of which are associated with dengue

disease severity) than serotype-specific clones. The majority of clones adopted a Tc0 phenotype, producing cytokines belonging to both the Tc1 and Tc2 paradigms. It has been hypothesised that the interplay between these cytokine groups has a part to play in determining the nature of the clinical presentation(92). Cytolytic ability at low concentrations of stimulating peptide correlated well with cytokine production. Cross-reactive cells producing high levels of cytokines maintained the ability to lyse target cells bearing peptide at concentrations one log lower than that at which serotype-specific clones lost their activity. Consistent with previous observations, cytolytic activity – whether assessed directly in chromium release assays or indirectly by CD107a/b expression – was sustained at lower peptide concentrations than was cytokine production(279).

These findings point to an obvious hypothesis. Primary infection leaves behind a pool of memory cells of varying affinities with the potential to act in a cross-reactive manner. In secondary infection these cells are rapidly expanded. Among them are a population of high avidity CTLs with correspondingly great effector function. These cells have the potential to become highly activated (as noted in observations of CD38 expression) producing large amounts of inflammatory cytokines in a manner deleterious to the host and contributing to pathogenesis. Others have noted the tendency of cross-reactive dengue specific CTL to produce increased amounts of such cytokines(290). They are however vulnerable to activation induced cell death and by convalescence many cross-reactive cells are lost even as a new memory population is generated as a result of the newly encountered virus. Such selective deletion of high avidity cells has been noted in leukaemia patients(255) and has been attributed to clonal exhaustion and activation induced cell death. This would be in keeping with

previous observations regarding the high level of dying A11 GTS tetramer positive cells in acute dengue patients(189).

These observations are clearly limited by the numbers involved. Work on frozen PBMC is limited by poor recovery of antigen specific cells, probably as a consequence of the very activation being sought. Samples stained with dengue tetramer before and after freezing have shown very different staining patterns with a big reduction in tetramer positive cells. This goes some way to explaining why only around 10% or less of stained samples had tetramer positive populations of any size. The assays described above should ideally be performed on fresh PBMC taken from a large number of patients shortly after blood letting. Such a study would hopefully contain within it sufficient samples with good staining that significance could be reached. Repeating these findings in that context would enable them to be applied to hypotheses relating to dengue pathogenesis with much more confidence.

One must also be cautious about immediately relating *ex vivo* observations made using tetramers specific for a single epitope and HLA type to the complex *in vivo* situation. Although undeniably immunodominant the HLA A11 response examined here is only one out of a presumed panoply of responses. It would perhaps be simplistic to expect to find a direct relationship between the response to a single epitope and clinical presentation in an acute viral infection such as dengue. One must be similarly reflective when applying information gleaned from work on T cell clones to a disease process. Clonal work provides much useful information but it is difficult to apply the findings from these artificial situations to an *in vivo* disease process with absolute confidence. Quite apart from any functional artefact that might occur as a

result of artificial stimulation, the clones generated in this study are likely to represent only a subset of the CD8+ T cells recognising the A11 GTS epitope. As was noted above, the majority of clones generated were cross-reactive and whilst this appears to reflect the *ex vivo* PBMC tetramer staining of patient MD1413 (figure 33) it does not appear to be the case with patient BC307 (figure 29). One cannot assume all serotype-specific clones would be of low affinity simply because the 3 generated in this study were. Neither can one assume the cross-reactive clones generated were a representative sample. It is possible that the differing cellular avidities for epitope result in the generation of a non-representative spectrum of clones (i.e. high affinity clones may be preferentially expanded by the *in vitro* stimulation used in the cloning process).

The CD8-non-binding tetramers described in this thesis may provide a means to answer two other questions logically proceeding from the above hypothesis: firstly "Is the size of the fraction of antigen-specific CTL showing high avidity related to the severity of clinical disease?" and secondly "Is this fraction disproportionately reduced by convalescence compared to the size of the antigen-specific CTL population as a whole?" The theory proposed above would lead one to hypothesise that the presence of a large high-avidity population would be associated with more severe disease as a consequence of their greater effector function, in particular cytokine production. Furthermore these high avidity cells should represent a significantly smaller fraction of the wild-type tetramer positive population by convalescence if, as suggested, they are selectively depleted by activation induced cell death. Unfortunately of 17 samples stained with both the wild-type and CD-non-binding tetramers only 1 showed good acute and convalescent staining. This sample did not show a significant drop in high-

avidity staining by convalescence. It is possible that by the time of clinical presentation the modulation of the T cell population is already well under way. The majority of patients have been unwell for a few days by the time of hospital admission and high avidity cells may be beginning to fall by this time point.

Another area of considerable interest is the role of cell mediated immunity in severe dengue disease in infants. Infants with severe disease would not be expected to have any dengue-specific T cell memory population and antibody-dependent enhancement provides an elegant account of the pathological process in this age group in particular. Yet as noted in the introduction, the permeability of an infant's microvasculature varies from that of older children and adults and it may be that the pathology of disease in the two age groups is not without its differences. It will fascinating to see the results of a study currently taking place in Viet Nam which aims to look at the immunology of infant disease, including antigen specific T cell responses.

CD4+ lymphocytes can demonstrate broad flaviviral cross-reactivity and are likely to be an important source of inflammatory cytokines and CD8+ T cell priming

CD4+ cells are important sources of cytokines and all the clones described in chapter 5 produced large amounts of type 1 cytokines in a cross-reactive manner. Clones described in previous studies have recognised this epitope, or one closely related to it, only in its DEN3 variant(211). The clones generated in this study showed cross-reactivity that extended beyond dengue, recognising variant peptides representing the corresponding epitope sequence of related flaviviruses: Japanese encephalitis, Yellow

fever and West Nile virus. The magnitude of the response varied with the extent of the homology shown to the dengue sequence but even a very different peptide such as that representing Yellow fever precipitated cytokine production and cytolysis. The nature of the effector response of dengue specific CD4+ T cells can be altered by the specific sequence of the presented epitope. It has been reported that the ratio of TNF-α to IFN-γ producing CD4+ cells from experimental vaccine recipients is higher after stimulation with antigen from heterologous dengue serotypes than homologous. These differing patterns of effector response may be significant in the immunopathogenesis of DHF(215).

The broader cross-reactivity seen with CD4+ cells is important. It is possible that CD4+ cells are capable of mediating cross-reactive responses to heterologous dengue virus precisely because they are able to tolerate small changes in their epitopes. Cells behaving in this way could produce an early cytokine response but also rapidly prime CD8+ cell populations during secondary dengue. There are many regions of the world where more than one flavivirus is prevalent and it has long been debated whether exposure to a different flavivirus (either as a vaccine or in "the wild") might modulate the immune response to dengue in a manner that might be either deleterious or beneficial to the host. Recipients of an experimental Japanese encephalitis vaccine have been shown to generate flavivirus cross-reactive CD4+ T cells that recognise the E protein(291). Despite many years of widespread Yellow fever vaccination there is (aside from a few case reports) little in the way of data indicating a specific association – good or bad – between previous flaviviral exposure and subsequent dengue disease.

CD107a/b is a potentially useful marker of CD4+ T cell cytolytic activity

CD107, which exists in 2 subtypes (a and b), is a glycolipid found in the membranes of lysosomes, degranulated platelets, activated neutrophils and T cells. It is a marker of degranulation and activation of CD8+ cells has been shown to cause a transient increase in surface expression of CD107 as granules release their contents onto the cell surface(237). Its presence correlates well with cytotoxic activity measured by traditional chromium release assays(292). Although it has also been used as a surrogate marker for the cytolytic activity of NK cells(289) it has not been widely used as a marker of CD4+ cell degranulation. All the CD4+ clones generated in this study demonstrated cytolytic activity in chromium release assays. In a manner similar to that seen with CD8+ T cell clones, CD107a/b surface expression was transiently increased after antigen stimulation of CD4+ T cell clones. It remains to be seen how useful CD107a/b staining of CD4+ cells might be in practice. Preliminary stains on peptide stimulated PBMCs did not result in CD107a/b positive populations (data not shown). This may reflect the relatively small antigen specific populations present in memory after an acute viral infection. The assay may be of more practical use in chronic viral infections such as HIV where the antigen specific population is much larger. Nonetheless it would be interesting to attempt to assess the *ex vivo* dengue specific CD107 expression among CD4+ cells in PBMC taken during acute disease, perhaps after a brief stimulation with a more physiological antigen spanning a more comprehensive portion of the proteome (e.g. viral lysate). Together with perforin staining such an experiment might shed some light on whether cytolytic CD4+ cells are present in an acute viral infection, as they are now known to be in chronic.

Conclusion

The clinical manifestation of dengue infection would appear to represent the sum of the interaction of several components of the immune system. Traditionally debate regarding the relative importance of humoral and cell-mediated immunity in the pathogenesis of severe dengue has been polarised, with proponents of different schools of thought tending to present their data(11) in a manner that implies mutual incompatibility[2]. However a growing body of opinion is adopting a more holistic view(290) and the data presented in this thesis supports this "third way".

This hypothesis could be summarised as follows: viral infection of target cells such as macrophages and monocytes is facilitated by antibody enhancement of secondary infection and perhaps the phenomenon of original antigenic sin leading to high viraemia and antigen loading of antigen-presenting cells (114, 115). Memory CD8+ CTL and CD4+ T cells from a previous infection that show cross-reactivity to this secondary viral serotype are expanded. CD4+ cells produce large amounts of inflammatory cytokines, priming CD8+ CTL and other components of the immune response. Both CD4+ cells and those CD8+ T cells showing high avidity for certain epitopes produce immunopathogenic levels of cytokines certain vasoactive members of which contribute to plasma leakage. The large antigen load combined with the high avidity of the cytotoxic T cells results in over-activation, cell death and clonal deletion. Serotype specific CTL of lower avidity are generated concurrently and survive into convalescence with the potential of producing cytokines in a therapeutic rather than immunopathogenic manner. It is likely that protective immunity is

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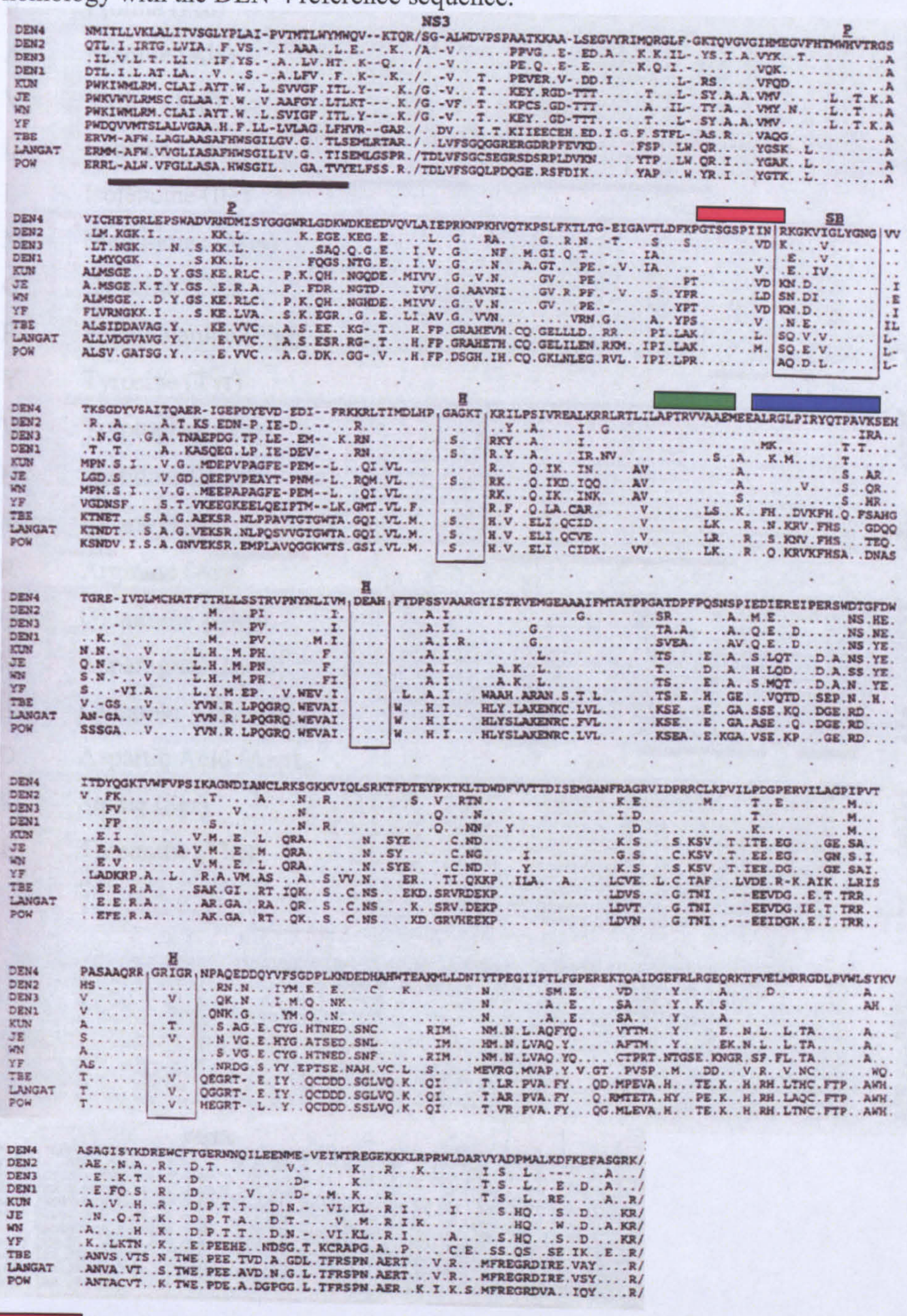
[2] Quote from reference 11: "It is evident that, in opposition to the view of Rothman and Ennis, cross-reactive T cells that are activated in response to secondary infection with a different serotype are not required to produce the high levels of cytokines that accompany severe DHF."

primarily antibody mediated – however it can be postulated that a *lack of immunopathogenesis*, as much as a degree of protection derives from these serotype-specific populations in patients with repeated exposure to dengue virus.

These findings have significant implications for understanding the role of virus-specific CD4+ and CD8+ T cells in immunity to dengue virus infection and in the pathogenesis of severe dengue disease. Those involved in vaccine development have rightly been concerned about avoiding disease enhancing effects as a consequence of an insufficiently broad neutralising antibody response. Of course an effective humoral response would greatly limit the response required of T cells. Yet it must not be forgotten that one the safest and most effective flaviviral vaccines, the 17D Yellow fever vaccine, elicits strong, long-lasting humoral *and* cellular immunity(110, 293). Neutralising antibodies develop in over 98% of recipients(294) and although less is known about the cellular response, virus specific CD8+ T cell responses are detectable up to 18 months after immunisation(295, 296). Most experts in the field believe the best hope for a dengue vaccine lies in the development of a tetravalent live-attenuated preparation. In the hunt for a vaccine that produces pan-serotype protective immunity without the risk of iatrogenic DHF it is surely prudent to consider not only the nature of the antibody response but the specificity, avidity and effector function of T cells elicited by dengue vaccine candidates – no matter what one's position in the immunopathological debate.

APPENDIX 1 – amino acid sequence of the flaviviral NS3 protein

The amino acid sequence of NS3 from the 4 dengue serotypes and 7 other flaviviruses with locations of the epitopes referred to in this thesis. "." indicates positions of homology with the DEN-4 reference sequence.



APPENDIX 2 – the single letter amino acid code

G	Glycine (Gly)
P	Proline (Pro)
A	Alanine (Ala)
V	Valine (Val)
L	Leucine (Leu)
I	Isoleucine (Ile)
M	Methionine (Met)
C	Cysteine (Cys)
F	Phenylalanine (Phe)
Y	Tyrosine (Tyr)
W	Tryptophan (Trp)
H	Histidine (His)
K	Lysine (Lys)
R	Arginine (Arg)
Q	Glutamine (Gln)
N	Asparagine (Asn)
E	Glutamic Acid (Glu)
D	Aspartic Acid (Asp)
S	Serine (Ser)
T	Threonine (Thr)

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